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(21) International Application Number: PCT/AU94/00742 (22) International Filing Date: 30 November 1994 (30.11.94) (30) Priority Data: PM 2705 30 November 1993 (30.11.93) AU PM 8234 16 September 1994 (16.09.94) AU (71) Applicant (for all designated States except US): THE UNIVERSITY OF QUEENSLAND [AU/AU]; St. Lucia, QLD 4072 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): MORTON, Halle [AU/AU]; 75 Orion Street, Coorparoo, QLD 4151 (AU). CAVANAGH, Alice, Christina [AU/AU]; 29 Anderson Avenue, Ashgrove, QLD 4060 (AU). (74) Agent: KELLY, Robin, Thomas; Fisher & Kelly, Level One, 349 Coronation Drive, Milton, QLD 4064 (AU).			(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  Published With international search report.
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TITLE

"ANTAGONISTS TO CHAPERONIN 10"

FIELD OF THE INVENTION

THIS INVENTION relates to antagonists to chaperonin 10  
5 otherwise known as cpn10.

PRIOR ART

Chaperonins belong to a wider class of molecular chaperones, molecules  
involved in post-translational folding, targeting and assembly of other proteins,  
but which do not themselves form part of the final assembled structure as  
10 discussed by Ellis *et al.*, 1991, Annu. Rev. Biochem. 60 321-347. Most  
molecular chaperones are "heat shock" or "stress" proteins (hsp); i.e. their  
production is induced or increased by a variety of cellular insults (such as  
metabolic disruption, oxygen radicals, inflammation, infection and  
transformation), heat being only one of the better studies stresses as reviewed  
15 by Lindquist *et al.*, 1988, Annu. Rev. Genet. 22 631-677. As well as these  
quantitative changes in specific protein levels, stress can induce the movement  
of constitutively produced stress proteins to different cellular compartments as  
referred to in the Lindquist reference mentioned above. The heat shock  
response is one of the most highly conserved genetic system known and the  
20 various heat shock protein families are among the most evolutionarily stable  
proteins in existence. As well as enabling cells to cope under adverse  
conditions, members of these families perform essential functions in normal  
cells.

There are two types of cpn molecules, cpn60 (monomeric  $M_r$   
25 ~60 000) and cpn10 (monomeric  $M_r$  ~10 000). Cpn60 has been studied  
extensively. It has been identified in all bacteria, mitochondria and plastids  
examined, and a cytoplasmic form, TCP-1, has been identified in eukaryotic  
cells; its presence on the surface of some cells has been reported, although  
this has been questioned in the Ellis reference referred to above and also in  
30 van Eden, 1991, Immunol. Reviews 121 5-28. Until very recently, cpn10 had  
been identified only in bacteria but structural and functional equivalents have  
now been found in chloroplasts (Bertsch *et al.*, 1992, Proceedings of the

National Academy of Sciences USA 89 8696-8700) and in rat (Hartman *et al.*, 1992, Proceedings of the National Academy of Sciences USA 89 3394-3398) and bovine liver mitochondria (Lubben *et al.*, 1990, Proceedings of the National Academy of Sciences USA 87 7683-7687).

5 Cpn60 and cpn10 interact functionally, in the presence of ATP, to mediate protein assembly. Instances of cpn10 acting independently of cpn60 have not yet been reported but cpn60, apparently acting alone, has been implicated in quite disparate events. For example, it is an immuno-dominant target of both antibody and T-cell responses during bacterial infections but, because the protein is so highly conserved, self reactivity is generated. Healthy individuals may use this self-recognition to eliminate transformed and infected autologous cells but defects in control of such recognition may lead to autoimmune disease as discussed by van Eden, 1991, Immunol. Reviews 121 5-28. Not surprisingly, cpn60 has been associated with conditions such as rheumatoid arthritis. There is thus a growing awareness that molecular chaperones, with their capacity to bind to and alter the conformation of a wide variety of polypeptides, may occupy key roles in cellular functions other than protein biogenesis. Reference may also be made to Hartman *et al.*, 1993, Proceedings of the National Academy of Sciences USA 90 2276-2280 which describes the stabilization of protein molecules using cpn10 and cpn60.

20 It can also be established that for mammalian cpn10's, there is a very close sequence homology. Thus, for example, the rat cpn10 molecule (Hartman *et al.*, 1992) has greater than 99% homology with the structure of bovine cpn10 reported in EMBL Data Base Directory under MT BTC PN10 which was submitted by J. E. Walker, MRC Lab. of Molecular Biology, Hills Road, Cambridge, UK: This has to be contrasted with bacterial cpn10's which have an average degree of homology with rat chaperonin 10 of only 34% (Hartman *et al.*, 1992).

#### EARLY PREGNANCY FACTOR (EPF)

30 EPF was first described as a pregnancy associated substance (Morton *et al.*, 1976, Proc. R. Soc. B. 193 413-419) and its discovery created considerable interest as it enabled the detection of a potential pregnancy within

6-24 hours of fertilisation. Initially EPF was assigned a role as an immuno-suppressant by virtue of its ability to release suppressor factors from lymphocytes (Rolfe *et al.*, 1988, Clin. exp. Immunol. 73 219-225). These suppressor factors depress the delayed type hypersensitivity reaction in mice  
5 and therefore might suppress a possibly maternal immune response against the antigenically alien fetus. More recent studies have shown that production of EPF is not confined to pregnancy. It is a product of primary and neoplastic cell proliferation and under these conditions acts as a growth factor (Quinn *et al.*, 1990, Clin. exp. Immunol. 80 100-108; Cancer Immunol. Immunother,  
10 1992, 34 265-271). EPF is also a product of platelet activation and it is proposed therefore that it may play a part in wound healing and skin repair (Cavanagh *et al.*, 1991, Journal Reproduction and Fertility 93, 355-365).

EPF is reviewed in detail by Morton *et al.*, 1992, Early Pregnancy Factor, Seminars in Reproductive Endocrinology 10 72-82. The  
15 site and regulation of EPF production is described, followed by the purification of EPF from platelets and the relationship of the purified product to EPF derived from other sources. This review also discusses certain aspects of the bioassay for EPF (i.e. the rosette inhibition test) including monitoring purification procedures and investigating sources of production. The  
20 biological activity of EPF is discussed and possible clinical applications of EPF and its antagonists are described.

Morton *et al.*, 1992, Reprod. Fertil Dev. 4 411-422 reviews previous publications describing the immuno suppressive and growth factor properties of EPF. The role of EPF in maintaining the pre-embryo is also  
25 discussed in this reference.

To date, the rosette inhibition test remains the only means of detecting EPF in complex biological mixtures (Morton *et al.*, 1976, Proc R Soc B 413-419). This assay is dependent on the original finding of Bach and Antoine, 1968, Nature (Lond) 217 658-659 that an immunosuppressive anti-  
30 lymphocyte serum (ALS) can inhibit spontaneous rosette formation *in vitro* between lymphocytes and heterologous red blood cells. A modification of the assay was introduced to detect EPF after it was demonstrated that

lymphocytes, preincubated in EPF, give a significantly higher rosette inhibition titre (RIT) with an ALS than do lymphocytes from the same donor without EPF as described in the 1976 reference above. This test has been described in detail in the above 1976 reference as well as in Morton *et al.*, 1987, in "In Current Topics in Developmental Biology" Vol 23 73-92, Academic Press, San Diego, but briefly it involves a cascade of events with EPF binding to lymphocytes and sequentially inducing the release of suppressor factors (Rolfe *et al.*, 1988, Clin. exp. Immunol. 73 219-225); (Rolfe *et al.*, 1989, Immunol. Cell Biol. 67 205-208).

10 In Athanasas-Platsis *et al.*, 1989, Journal Reproduction and Fertility 87 495-502 and Athanasas-Platsis *et al.*, 1991, Journal Reproduction and Fertility 92 443-451, there is described the production of monoclonal and polyclonal antibodies to EPF and passive immunization of pregnant mice with these antibodies which causes loss of embryonic viability. These studies  
15 established that EPF is necessary for the successful establishment of pregnancy.

In Quinn *et al.*, 1990, Clin. exp. Immunol. 80 100-108, it is proposed that EPF is a growth regulated product of cultured tumour and transformed cells. These cells are also dependent upon EPF for continued  
20 growth i.e. EPF acts in an autocrine mode.

It has been established that EPF plays a role in promoting tumour growth since the growth of tumour cells can be significantly retarded by anti-EPF mAbs. In addition this reference suggests that hybridomas producing high affinity anti-EPF antibodies may be inherently unstable.

25 In Quinn *et al.*, 1992, Cancer Immunol. Immunother. 34 265-271, there is also described the effect of monoclonal antibodies (mAbs) to EPF on the *in vivo* growth of transplantable murine tumours. The main thrust of this reference is that neutralisation of EPF retards tumour growth *in vivo*.

It is clear from the above Quinn *et al.* 1992 reference that when  
30 cancer is in the very early stage of growth, neutralisation of EPF by anti-EPF mAb will prevent its development. However, once the cancer becomes established, treatment with these mAbs will retard but not entirely destroy the

tumour.

Other references in regard to the role of EPF in tumour growth include Quinn, 1991, Immunol. Cell Biol. 69 1-6 and Quinn, K. A. in a PhD thesis entitled "Early pregnancy factor: a novel factor involved in cell proliferation"<sup>11</sup> from the University of Queensland in Australia in 1991.

From the foregoing it therefore will be appreciated that EPF is indicative of and necessary for embryonic well being and has immunosuppressive properties mediated through induction of at least two suppressor factors. Recent studies indicate the importance of EPF as a growth regulator of transformed, neoplastic and normal cells while its presence in platelets implies a role in inflammation and wound healing.

A molecule possessing this exceptional combination of properties has great significance but to date the structure of this molecule has proved to be elusive.

It has now been established unexpectedly that mammalian chaperonin 10 has an amino acid sequence which is the same as early pregnancy factor (EPF).

#### SUMMARY OF THE INVENTION

In one aspect, the invention provides for the production of antibodies specific for cpn10, cpn10 derivatives or peptides derived from cpn10.

Another aspect of the invention is the use of anti-cpn10 antibodies or other antagonists. The use of the anti-cpn10 antibodies or other antagonists may include growth suppressing activity or an immune enhancing activity.

The present invention includes within its scope the use of (i) monoclonal and/or polyclonal antibodies to eucaryotic, procaryotic, recombinant or synthetic cpn10 or modifications or fragments thereof, or engineered constructs based on the active site or centre of these antibodies, or (ii) antagonists of cpn10, based on modifications of the structure of the molecule or fragments thereof.



## EXPERIMENTAL

A. PURIFICATION OF CPN10 AND ANTIBODY PRODUCTION

- 5 (i) *Purification of Human EPF from Human Blood Platelets*  
(FIGS. 1a, 1b, 1c, 1d)

Extraction

Platelet concentrates (from the Blood Bank), up to 7 days clinically outdated, were washed with Tyrodes buffer, following the techniques described in Methods in Enzymology, 1989, 169 7-11, snap frozen in liquid N<sub>2</sub> and stored at -70°C.

Immediately prior to purification, approximately 100 washed platelet units were thawed in a boiling water bath, then held at 75-85°C for 15 min with continuous, gentle stirring. After cooling on ice, cellular debris was removed by centrifugation (8000 g, 20 min, 4°C) and the pellet extracted twice by homogenisation in 0.05 M-acetic acid/0.1 M-NaCl/0.1 mg/ml sodium azide pH 3.0 followed by centrifugation (8 000 g, 15 min 4°C). The three supernatants were pooled giving a total extract volume of 500-600 ml.

Ion-exchange chromatography

20 This extract from 100 platelet units was adjusted to pH 3.0 with conc. HCl and stirred gently, overnight, 4°C, with 250 ml SP-Sephadex C-25 (Pharmacia-LKB), previously swollen and equilibrated with 0.05 M-acetic acid/0.1 M-NaCl pH 3.0. The gel was then packed into a column washed with 20 vol of the same buffer and eluted with 5 vol 0.5 M-sodium phosphate buffer/0.05 M-NaCl pH 7.5. The gel was then discarded.

Affinity chromatography

30 The SP-Sephadex eluate was adjusted to pH 6.3-6.4 with conc. HCl and applied to a column of Heparin-Sepharose CL-6B (2.5 x 7.5cm; Pharmacia-LKB) previously equilibrated with 0.05 M-sodium phosphate buffer/0.05 M-NaCl pH 6.3. The column was then washed with 5 vol of the same buffer and eluted with 5 vol 0.05 M-Tris-HCl/5 mM-CaCl<sub>2</sub>/0.2 M-NaCl pH 7.5, applied in the reverse direction to that used for sample application.

High performance hydrophobic interaction chromatography (HIC-h.p.l.c.)

Solid  $(\text{NH}_4)_2\text{SO}_4$  was added to the Heparin-Sepharose eluate to a final concentration of 2 M and, after passage through an  $0.45 \mu\text{m}$  filter, the sample was pumped through a dedicated solvent line onto a TSK Phenyl 5PW column ( $7.5 \times 75\text{mm}$ , Pharmacia-LKB), previously equilibrated with 0.1 M-Tris-HCl pH 7.0/5mM  $\text{CaCl}_2$ /2 M- $(\text{NH}_4)_2\text{SO}_4$ . The column was washed with 10 vol of the same buffer and eluted with a 50 min linear gradient from this buffer to 0.1 M-Tris-HCl pH 7.0/5 mM- $\text{CaCl}_2$ /10% acetonitrile. (FIG. 1a)

RP-h.p.l.c.-1

Active HIC-h.p.l.c. fractions were pooled, then fractionated on a  $\text{C}_3$  column (Ultrapore RPSC, Beckman Instruments) using a solvent system consisting of A, 0.04 M Tris/HCl pH 7.0/5 mM- $\text{CaCl}_2$  and B, 0.04 M-Tris/HCl pH 7.0/5 mM- $\text{CaCl}_2$ /80% (v/v) acetonitrile. The column was equilibrated with Solvent A prior to sample application, after which it was washed with 5 vol solvent A and eluted with a 30 min linear gradient from this solvent to 75% solvent B. (FIG. 1b)

RP-h.p.l.c.2

Active fractions from RP-h.p.l.c.-1 of several 100 unit platelet preparations were pooled, EDTA and DTT added to a final concentration of 20mM and 1 mM respectively and the mixture allowed to stand for 0.5-1 h,  $4^\circ\text{C}$ . Following dilution with 2 vol solvent A, it was applied to a  $\text{C}_3$  column, dedicated to this and subsequent steps, and fractionated as described for RP-h.p.l.c.-1, but omitting  $\text{CaCl}_2$ . (FIG. 1c)

Rph.p.l.c.3

Active fractions from RP-h.p.l.c.-2 were pooled, trifluoroacetic acid (TFA) added to a final concentration of 0.1% and, following dilution with 2 vol 0.1% TFA, the mixture was applied to the  $\text{C}_3$  column, which had been equilibrated previously with 0.1% TFA. The column was then eluted with a 30 min linear gradient from this solvent to 60% (v/v) acetonitrile/0.1% TFA, followed by a 3 min linear gradient to 90% (v/v) acetonitrile/0.1% TFA. Active fractions were pooled. (FIG. 1d)

One unit represents platelets from a single blood donation which

is approximately 500 ml. The "active fractions" were fractions active in the rosette inhibition test.

Purification of EPF from other sources

The purification of cpn10 from various sources have been summarised in Cavanagh *et al.*, 1994, Eur. J. Biochem. 222 551-560.

Biological activity followed the same pattern throughout the complex purification scheme described above for human platelets. Furthermore the final active fraction from all sources was bound specifically by an immobilised monoclonal anti-EPF and could be recovered virtually quantitatively (see FIG. 1e).

These studies are important for several reasons:-

- A. The biochemical and immunological similarity observed with all these materials provides strong evidence that the bioassay is detecting a single substance or closely related family of substances acting in diverse biological situations.
- B. The active agents purified from all of these materials are from several to many orders of magnitude more potent than virtually all of the substances previously reported to be EPF summarised in the abovementioned Morton *et al.*, 1992 reference. This confirms our surmise, based on detailed analysis of the EPF bioassay as discussed above, that activity associated with most putative EPF preparations must reflect the presence of a very minor contaminant.
- C. The only source materials providing sufficient EPF to study at the protein (as opposed to activity) level were platelets and regenerating liver, yielding, respectively, an average of 15  $\mu$ g per 100 units (equivalent to ~ 50 litre blood) and 5  $\mu$ g per 40 g tissue (liver remnant from 6 rats). It is immediately apparent that far more EPF is present within the cell than appears in the extracellular space: nevertheless, accumulated knowledge of the biology of EPF (reviewed recently in the abovementioned Morton *et al.* 1992 reference) indicates that this extracellular

appearance is not fortuitous.

Human platelet-derived EPF, being most abundant, has been studied in some detail. On SDS-PAGE, it ran as a single band of  $M_r$  approx. 8 500, coincident with biological activity (see FIG. 2a); EPF from  
5 regenerating rat liver exhibited identical behaviour. Mass spectrometry of the platelet material provided an accurate and precise determination of molecular mass  $10\,843.5 \pm 2$  Da, along with definitive evidence of the high degree of homogeneity of the preparation (see FIG. 2b). Following attempts at Edman  
10 degradation, which indicated that the molecule is N-blocked, proteolytic cleavage of approx. 4 nmol EPF was undertaken. Resultant peptide fragments were separated by reversed-phase HPLC and subjected to sequencing by Edman degradation. Three areas of sequence containing 12 (fragment 1), 27 (fragment 2) and 33 (fragment 3) residues were found to correspond with  
15 residues 7 to 18, 27 - 53 and 69 - 101 (the C-terminus) in rat mitochondrial cpn10. In fragment 2, residue 52 was different (S in cpn10, G in rat cpn10; this change alone could account for human cpn10 being 30 Da larger than rat cpn10). All other residues were identical, consistent with the highly conserved nature of chaperonins (see FIG. 2c).

Since confirming sequence identity between EPF and cpn10,  
20 several studies of functional relationship have been performed, using rat mitochondrial cpn10, *E. coli* cpn10 (known as *groES*) and *E. coli* cpn60 (*groEL*). First it has been demonstrated that cpn10 can act as EPF. Rat cpn10 was tested in the EPF bioassay and found to be positive over the range of dilutions expected; this activity could be neutralised by monoclonal  
25 antibodies to EPF. Interestingly, *E. coli* cpn10, which is ~ 40% homologous with rat cpn10, exhibited no activity in the bioassay. This is consistent with the observation that *E. coli* conditioned medium is not active in the EPF bioassay, while medium conditioned by all mammalian cell lines tested, as well as by yeast cells is active. Cpn60 was inactive in the bioassay and had no  
30 effect upon the activity of EPF. It was then shown that EPF can act as cpn10. EPF was mixed with cpn60, in the presence or absence of ATP, and the mixture fractionated on a TSK G3000SW gel permeation column; resultant

fractions were analysed by SDS-PAGE. Cpn60 is a decatetramer and elutes in the excluded volume of this column (exclusion limit 300 000). In the presence of ATP, but not in its absence, EPF also appears in this fraction, demonstrating formation of a stable complex with cpn60. This fraction was  
5 active in the EPF bioassay but the equivalent fraction from the experiment without ATP (where EPF did not associate with cpn60) was not (see FIG. 3a). Thus EPF and cpn10 activity reside in the same molecule.

These investigations provide unequivocal evidence that platelet-derived EPF is a structural and functional homologue of cpn10; the  
10 relationship between cpn10 and activity in the rosette inhibition test was then examined (FIG. 3b). In the presence, but not in the absence of ATP, immobilised cpn60 could remove all activity from the archetypal source material, pregnancy serum and activity could be recovered by removing ATP from the immobilised complex. As with the experiment described in FIG. 3a,  
15 this requirement for ATP demonstrates the specificity of the interaction between cpn60 and the active moiety; cpn10 is thus the molecular entity initiating response in the EPF bioassay.

Identification of EPF as a cpn10 has been a major step forward in research on this subject and helps to explain many of the findings that have  
20 been made to date. Criticism has been raised against claims that EPF production occurs in such a wide variety of biological situations e.g. pre-and post-implantation pregnancy, primary and tumour cell proliferation and platelet activation. In its role as a hsp (heat stress protein), these are all conditions in which the rapid onset of EPF production would be expected. Functions of  
25 hsp's that are vital to the survival of cells are intracellular as shown in the Linquist *et al.* reference above. In contrast, the activity of EPF described to date is extracellular; for example, it appears in serum of mice within 4 to 6 hours after mating as discussed in Morton *et al.*, 1987, Current Topics in Development Biology, Vol 23 73-92 and 4 to 8 hours after partial hepatectomy  
30 in rats as shown in the Quinn PhD thesis (1991). We have shown that EPF can act in an autocrine mode as discussed in the Quinn *et al.*, 1990 reference referred to above or exocrine mode as discussed in the Rolfe *et al.* 1988

referred to above; these are not roles previously described for hsp's.

It will also be appreciated that since the structure of EPF is now known, it can be produced in commercial quantities by any suitable technique such as by recombinant DNA techniques or by chemical synthesis.

## 5 (ii) *PRODUCTION OF ANTI-CPN10-DERIVED PEPTIDE*

Described here are the methods used and results encountered in the production of anti-cpn10-derived peptide. Peptides of cpn10 may include the following amino acid sequences:-

- (i) AGQAFRKFLPL;
- 10 (ii) Ac-AGQAFRKFLPL;
- (iii) EKSQGKVLQAT
- (iv) A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub>;
- (v) AGQAFRKFLPLA<sub>2</sub>;
- (vi) A<sub>1</sub>AGQAFRKFLPL;
- 15 (vii) Ac-A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub>;
- (viii) Ac-AGQAFRKFLPLA<sub>2</sub>;
- (ix) Ac-A<sub>1</sub>AGQAFRKFLPL;
- (x) A<sub>1</sub>EKSQGKVLQATA<sub>2</sub>;
- (xi) EKSQGKVLQATA<sub>2</sub>;
- 20 (xii) A<sub>1</sub>EKSQGKVLQAT;

wherein A<sub>1</sub> and A<sub>2</sub> are amino acid sequences which may be added to one or each end of molecules (i) through (xii) and Ac is acetyl.

Anti-cpn10-derived peptides antibodies may include antibodies raised against any one of the aforementioned amino acid sequences (i) - (xii).  
25 As an example, antibodies have been raised against an N-terminal fragment (Ac-AGQAFRKFLPLC) and an internal fragment (EKSQGKVLQATC).

It will be appreciated in the abovementioned peptides that such peptides may include a single amino acid addition, deletion or substitution and the invention also includes antibodies raised against such peptides.

## 30 METHODS

### *Synthesis of cpn10 derived peptides*

Peptides were synthesized to correspond with an N-terminal

fragment (N-peptide i.e. Ac - AGQAFRKFLPLC) and an internal fragment (I-peptide i.e. EKSQGKVLQATC).

#### *Conjugation of peptides to ovalbumin*

Peptides were conjugated to ovalbumin by the hetero-bifunctional reagent SPDP, following manufacturer's instructions (Pharmacia-LKB  
5 Biotechnology, Uppsala, Sweden).

#### *Immunisation schedules*

Adult outbred New Zealand rabbits were immunised with one of the conjugates in 4 x weekly injections followed by several monthly boosts.

10 For injection, the antigen was dialysed into 0.9% saline (Mr 12-15000 cut off dialysis tubing, Visking, Union Carbide, IL, USA) and emulsified with an equal volume of Freund's adjuvant (complete for the first injection, incomplete thereafter). Immunisations were via the s.c. route. Table 1 shows the amount of antigen injected.

#### 15 *Screening of antiserum*

Antisera were tested in an ELISA against the relevant antigens (viz. I-peptide or N-peptide; ovalbumin) (5 mg/ml). Bound IgG was detected by the biotin-streptavidin system (Amersham) with o-phenylene diamine as substrate. Absorbance was read at 492 nm.

20 Anti-N-peptide Abs were also tested in parallel with anti-EPF Abs #810 and #816 (Athanasios-Platsis *et al.*, 1989, J. Reprod. Fert. 87 495-502) against platelet derived EPF (1 mg/ml) (Cavanagh *et al.*, 1994, Eur. J. Biochem. 222 551-560) and N-peptide (5 mg/ml).

#### *Purification of antibodies*

25 IgG was purified from serum by affinity chromatography. N and I peptides, and ovalbumin were coupled separately to a HiTrap<sup>TM</sup> affinity column (HiTrap NHS-activated 1ml, Pharmacia-LKB) following the manufacturer's instructions. Each column was equilibrated with 0.05 NaPi-0.5M NaCl, pH 7.4, and the relevant antiserum applied, according to the manufacturer's  
30 instructions. After extensive washing with equilibration buffer, bound rabbit IgG was eluted by 0.2M glycine, pH 2.5. The pH of the eluate was adjusted with 2M Tris to approximately 7.4.

The purity of the Abs in the eluted fractions was determined by SDS-PAGE, then the strongest fractions pooled.

#### *Protein estimation*

Protein (IgG) was determined by the method of Lowry (Lowry  
5 *et al.*, 1951, J. Biol. Chem. 193 265-275), using a commercial preparation of Folin and Ciocalteu's reagent (Stansens, Qld, Australia). The standard curve was constructed with a purified rabbit IgG preparation (20 mg/ml; Silenus, Hawthorne, Australia).

#### RESULTS

10 The ELISA screening of the antibodies provided some interesting results.

The anti-peptides Abs titre decreased even with repeated boosting (FIG. 4a, 4b), while the production of anti-ovalbumin control Abs gave a normal response (FIG. 4c). Note that the titre of anti-ovalbumin Abs  
15 in rabbits immunised with the peptide conjugates (FIG. 4a, 4b) decreased as well.

Cross reactivity studies are shown in FIG. 5.

The titre of affinity purified Abs was determined by ELISA against the immunising peptide conjugated to bovine serum albumin (BSA).  
20 This test also demonstrated the efficiency of the procedure. Results are shown in Table 2.

These Ab preparations were shown in SDS-polyacrylamide gel electrophoresis to be approximately 95% pure.

#### CONCLUSION

25 The decreasing titre of Abs during the immunising schedule suggests a role for cpn10 in the proliferation of B cell clones. The instability of antibody-producing B cell clones which produce anti-EPF antibodies has been previously described. The pattern of anti-EPF antibody production was as described above, with maximum titres obtained 5 weeks after the initial  
30 immunisation and then falling despite frequent boosting. *In vitro*, hybridomas producing anti-EPF antibodies were inherently unstable (Quinn *et al.*, 1990, Clin. Exp. Immunol. 80 100-108). The difficulty in making a stable cell line



of a hybridoma which produces an anti-EPF/cpn10 antibody may be due to the autocrine action of EPF/cpn10 in cell proliferation, i.e. antibodies neutralize EPF/cpn10 which proliferating cells produce for their own growth advantage.

(iii) *PREPARATION OF ANTIBODIES TO RECOMBINANT CPN10*

5

*Cloning of human cDNA encoding cpn10 and production of cpn10*

Production for commercial use may be obtained by inserting a mammalian cpn10 gene, preferably a human cDNA cpn10 gene, into a suitable vector such as plasmids from the pGEX system, and pET system expressing the encoded mammalian cpn10 and purifying the recombinant cpn10.

10

*Abbreviations*

	ANGIS	Australian National Genomic Information Service
	bp	base pair
15	BSA	bovine serum albumin
	cDNA	complementary DNA
	cpn10	Chaperonin 10
	DNA	deoxyribonucleic acid
	<i>E. coli</i>	<i>Escherichia coli</i>
20	GSH	glutathione (reduced form)
	GST	glutathione-S-transferase
	LB	Luria-Bertani Broth
	M	Molar
	ORF	open reading frame
25	PCR	polymerase chain reaction
	rEPF	recombinant Early Pregnancy Factor
	RSP	reverse sequencing primer
	SDS	sodium dodecyl sulphate
	SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel
30		electrophoresis
	Tris	Tris(hydroxymethyl)aminomethane
	USP	universal sequencing primer

### Materials and Methods

#### Cloning of Human cpn10 Open Reading Frame

Polymerase chain reaction (PCR) was used to initially amplify part of the ORF (274 bp) of the human cpn10 cDNA from a melanoma cell line A2058 cDNA lambda library (Stratagene). A degenerate cpn10 amplimer (P1) was designed from the amino acid sequence VLDDKDYFL corresponding to amino acid residues 83-91 of human cpn10. The primer P1 has the sequence 5' ARRAARTARTCYTTRTCRTC 3' where R is A or G and Y is C or T. The reverse sequencing primer (RSP) was used for PCR amplification (the non-specific primer) as well as for sequencing DNA constructs and has the sequence 5' CAGGAAACAGCTATGAC 3'. The universal sequencing primer has the sequence 5' GTAAAACGACGGCCAGT 3'. PCR amplification of the phage library was achieved using a non-specific upstream amplimer (RSP) and P1, each at 0.5  $\mu$ M final concentration, 1.5 mM MgCl<sub>2</sub> (Pharmacia Biotech), 1X polymerase buffer (Boehringer Mannheim) and 5 units of *Thermus aquaticus* DNA polymerase (Boehringer Mannheim) in a final volume of 50  $\mu$ L. For 30 cycles, the parameters were: denaturation at 94°C for 1 min, annealing at 40°C for 30 sec and extension at 72°C for 3 min. A final extension at 72°C for 7 min was followed by a soak cycle at 4°C for 10 min. An aliquot of 1  $\mu$ L was reamplified under the same conditions to increase the copy number.

Two cpn10 specific amplimers encompassing the open reading frame were designed. The upstream primer P2, 5'-GCGCGGATCCATGGCAGGACAAGCGTTTAG-3', was designed from the sequence of the initial PCR fragment. The downstream primer P3, 5'-ATATGAATTCAGTCTACGTACTTTCC-3' was designed from sequence obtained from the Expressed Sequence Tag database via ANGIS (Accession No. HUM00TB037). A 319 bp fragment was amplified from the phage library using the same reaction and cycling conditions as above except the annealing temperature was 50°C.

#### DNA constructs and Analysis

All restriction enzyme digests of PCR products and vectors were

performed according to Sambrook *et al.* (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, NY) using restriction enzymes and their buffers obtained from Boehringer Mannheim. The initial PCR fragment was digested with *Eco* R1 and ligated (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, NY) into the *Eco* R1 and *Sma* I sites of pBluescript KS(+) (Stratagene) creating the plasmid pRM1 (FIG. 6a; partial *cpn10* insert 274 bp). The 319 bp product was digested with *Bam* HI and *Eco* R1 and initially cloned into the expression plasmid pGEX-2T (Pharmacia Biotech) creating the plasmid pRM2 (FIG. 6b). To confirm its identity, the *Bam* HI-*Eco* R1 fragment was subcloned into pBluescript (SK+) (pRM3; FIG. 6c) and sequenced. DNA was analysed on 0.8-1.0% (w/v) agarose gels containing ethidium bromide and after electrophoresis was viewed under UV illumination.

#### 15 Transformation of *E. coli*

Competent *E. coli* DH5 $\alpha$  cells (100  $\mu$ L) were transformed with the plasmids by the heat pulse method (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, NY). The mixture of cells and DNA (10-100 ng) was placed on ice for 30 min and heat pulsed for exactly 2 min at 42°C and placed back on ice for 2 min. The cells were allowed to recover at 37°C with shaking for 1 hr after the addition of 0.9 mL of LB. A 100  $\mu$ L aliquot was plated onto LB agar plates supplemented with Ampicillin at a final concentration of 100  $\mu$ g/mL. After incubation overnight at 37°C, random colonies were selected for further investigation.

#### 25 DNA sequence determination

Restriction fragments of the PCR products were cloned into pBluescript and sequenced in both orientations by the dideoxy chain-termination method using the T7 Polymerase Kit according to the manufacturer's instructions (Pharmacia Biotech). Approximately 2  $\mu$ g of plasmid DNA was denatured, ethanol precipitated and annealed to either the USP, RSP or P3. The sequencing reactions were electrophoresed on a 8%

acrylamide/46% urea gel. After fixing and drying, X-ray film was exposed to the gel overnight and developed.

Expression and purification of recombinant cpn10 in E. coli

Clones transformed with pRM2 were screened for expression of the Glutathione-S-transferase fusion protein on a small culture scale (2 ml) according to methods described by Smith *et al.* (Smith *et al.*, 1988, Gene 67 (1) 31-40). An overnight culture was diluted, induced to express the fusion protein by the addition of IPTG to 0.1 mM and grown at 37°C for several hours. The cells were pelleted, lysed in PBS/0.1% Triton X-100 and the lysate mixed with 50% Glutathione-Agarose beads (Sigma Chemical Company). The recombinant fusion protein was eluted from the affinity beads by boiling in SDS loading buffer. An aliquot of the sample was run on a 10% SDS-PAGE gel. The gel was fixed and then stained with Coomassie blue. After confirming the expression of the fusion protein the purification of rcpn10 from the GST moiety was undertaken on a larger scale.

Cells were grown and induced as above, the cell pellet resuspended in PBS, sonicated (output level 4, 50% duty cycle, 2 x 30 sec) and the cell lysate stored at -30°C. Lysate from 10 litre cell culture was thawed and rcpn10 isolated by similar techniques to those used by Gearing *et al.* (Gearing *et al.*, 1989, Biotechnology 7 1157-1161) for isolation of rLIF. Briefly, Triton X-100 was added to a final concentration of 0.1% and cellular debris removed by centrifugation (15 min, 15000 rpm, 4°C). Ten ml glutathione-Sepharose 4 B gel (Pharmacia - LKB Biotechnology) was added to the supernatant and the slurry mixed for 2 hr, 4°C. The gel was pelleted, washed x 5 with 50 ml PBS/0.1% Triton X-100 once with 50 ml 0.05 M Tris-HCl pH 8.0/0.15 M NaCl and once with 0.05M Tris-HCl pH 8.0/0.15 M NaCl/2.5 mM CaCl<sub>2</sub>. The gel was resuspended in 4 ml of 0.05 M Tris-HCl pH 8.0/0.15 M NaCl/2.5 mM CaCl<sub>2</sub> buffer, 1000 units thrombin (Sigma T6884) added and the slurry was mixed in a shaking waterbath for 1 hr, 37°C. The gel was pelleted, the supernatant retained, and the gel was then washed with 3 x 4 ml 0.05 M Tris-HCl pH 8.0/0.15 M NaCl. These washes and the first supernatant, which contain the rcpn10, were pooled, yielding 4-5 mg

recombinant protein. Additional rcpn10, which was non-specifically bound to the gel, was recovered as follows. Four ml 0.05 M Tris-HCl pH 8.0/2 M NaCl was added and the slurry mixed for 2 hr, 4°C.

After pelleting, the gel was washed with 3 x 2 ml of this 0.05 M Tris-HCl pH 8.0/2 M NaCl buffer, the washes pooled with the first supernatant, yielding a further approximately 1 mg rcpn10. Protein concentrations were estimated by the method of Lowry *et al.* (Lowry *et al.*, 1951, J. Biol. Chem. 193 265-275); proteins were analysed by SDS-PAGE using 15% Tris-Tricine gels (Schagger *et al.*, 1987, Anal. Biochem. 166 368-379).

The recombinant cpn10 has two additional amino acids at the N terminus. The N terminus of the recombinant protein is Gly-Ser-Ala whereas the N-terminus of native protein is Ac-Ala. The amino acid sequence of the recombinant cpn10 is as follows:

GSAGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVEA  
VGSGSKGKGGEIQPVSVKEGDKVLLPEYGGTKVVLDDKDYFLFRDGDIL  
GKYVD.

Antibodies were raised against the GST:rcpn10 fusion protein.

Antibodies against the recombinant protein were raised in rabbits using the same schedule described for producing anti-peptide antibodies. Approximately 10 µg protein was used for each injection. Rabbit serum was screened for anti-cpn10 antibodies by ELISA, using the technique described for screening anti-peptide antibodies with the exception that plates were coated initially with cpn10 (5 µg/ml). The antibody (Ab) titres against cpn10 and against the whole fusion protein (in this case, GST:rcpn10, 5 µg/ml, was bound to the plate) in serum of rabbit #42 are shown in FIG. 7. Titration of a serum sample against cpn10, taken from this rabbit after the 4th booster does, is illustrated in FIG. 8.

#### B. PREGNANCY TERMINATION

In another aspect of the invention, pregnancy may be terminated by administration of antibodies specific for cpn10 to a pregnant subject. The antibodies may be raised against cpn10 or derivatives therefrom. The

administration of these antibodies preferably occurs at the pre-implantation stage (1-2 cell stage) or at the peri-implantation stage. Pregnancy termination with anti-cpn10 antibodies is demonstrated below by way of example in a mouse model system. The mouse model system is by way of example only and the method is not limited to mice. The method may be applied to any suitable mammalian species including man.

(i) **ANTIBODIES RAISED AGAINST CPN10 PEPTIDES  
TERMINATES PREGNACY AT PRE-IMPLANTATION  
STAGE**

10 ***Anti-cpn10 antibodies***

The preparation and characterisation of these antibodies have been described. In these experiments, antibodies used were those prepared against the N-terminal peptide (cpnN) and an internal peptide (cpnI); cpnN and cpnI are active in the rosette inhibition test. IgG was precipitated from anti-serum by 45% ammonium sulphate and the concentration determined by Lowry and gel electrophoresis. The IgG preparations were tested in an ELISA against the immunising peptide, conjugated to bovine serum albumin. The preparations were also tested for their ability to neutralise activity in mouse pregnancy serum. Various concentrations of antibody were incubated with an equal volume of mouse serum then the mixtures tested for activity in the rosette inhibition test. The lowest concentration of antibody that could completely neutralise EPF activity was determined (see Cavanagh *et al.*, 1994, Eur. J. Biochem. 222 551-560). Ten pg anti-N-peptide Ab neutralised the activity of 1 ml of pregnancy serum while 4 ng anti-I-peptide was needed for complete neutralisation.

25 ***Passive Immunisation***

Mature outbred male and female Quackenbush mice were caged in pairs at 7.30 a.m. and separated at 8.30 a.m. Female mice with vaginal plugs were injected with anti-N-peptide/ovalbumin, anti-I-peptide/ovalbumin or anti-ovalbumin IgG preparations at 9.00 a.m. and 5.00 p.m. on days 1 (day of mating) and 2 of pregnancy. The dose of specific IgG injected in the 2 dose regimen was estimated as approximately 1 mg/mouse/day. On day 7, mice

were euthanased with CO<sub>2</sub>, uteri examined for implanted embryos and the number of corpora lutea (CL) counted. In each group, the number of embryos/CL in the mice treated with the test IgG was compared with the number receiving the same dose of control IgG ( $\chi^2$  test).

## 5 *Results*

The results, shown in Table 3, clearly demonstrate that neutralisation of activity in pregnancy serum can adversely affect embryonic viability in the early stages of pregnancy. The ability of antibodies to neutralise cpn10 activity in the rosette inhibition test is an *in vitro* monitor of their ability *in vivo* to adversely affect pregnancy.

## 10 C. CANCERS AND TUMOURS

A further aspect of the invention is the suppression of growth of abnormal cells by the administration of antagonists of cpn10 to a subject. Said abnormal cells or aberrant growth of normal cells include tumour or cancer cells; aberrant growth of normal cells includes diseases such as in psoriasis or Reiter's syndrome. Tumour cells include those from both benign and malignant growths. Cells from malignant diseases such as solid tumours and haematological cancers may also be included. An example of the suppressing effect of tumour cell growth is demonstrated by experiments with murine B16 melanoma and MCA-2 fibrosarcoma cell lines.

### 20 (i) ***EFFECT OF ANTI-CPN10-DERIVED PEPTIDES ANTIBODIES (ABS) ON THE GROWTH OF TUMOUR CELLS IN VITRO***

#### INTRODUCTION

25 The following studies investigate the possibility that cpn10, produced by tumour cells *in vitro*, is also required by these cells for their continued growth.

#### METHODS

##### *Cell culture*

30 Cell lines were cultured under standard conditions in basal medium, Dulbecco's modification of Eagle's medium (DMEM; ICN Biochemicals Australasia Pty. Ltd., Australia), supplemented with 10% foetal

calf serum (FCS, ICN) 20mM glutamine (ICN) and antibiotics [100 µg/ml streptomycin (ICN), 100 U/ml penicillin (CSL, Melbourne, Australia)], at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

Cells were maintained in the logarithmic (log) phase of growth.

5 Monolayers were dissociated, after washing in serum-free medium, by a short exposure, at 37°C, to a solution of 0.1% w/v Trypsin and 0.02% w/v versene in calcium and magnesium free balanced salt solution. The action of the trypsin was neutralised with the addition of medium containing 2% v/v FCS and the cells were recovered by centrifuging 200 g for 5 min, washed a further  
10 two times in serum free medium, after which they were seeded into culture dishes or 96 well plates (NUNC).

Stocks of cell lines were maintained frozen in liquid N<sub>2</sub> at all times.

*Preparation of anti-peptides Abs for co-culture experiments*

15 Affinity purified anti-N Abs, anti-I Abs and anti-ovalbumin Abs (control antibodies) were exchanged into DMEM and adjusted to a final concentration of 1 mg/ml. The preparations were sterilised by passage through a 0.2 µm cut-off filter (Minisart, Sartorius GmbH, Goettingen, Germany). As a control medium, DMEM alone was similarly treated.

20 *Co-culture of tumour cells with anti-peptides Abs*

The murine B16 melanoma and MCA-2 fibrosarcoma cell lines were studied. The cells (10<sup>4</sup>) were seeded in triplicate, in 0.2 ml culture medium (DMEM + 10% FCS (heat inactivated) containing doses of anti-peptide Abs, or control Ab, in the range 62.5 - 500 µg Ab/ml (final  
25 concentration). Cells were similarly seeded into filtered medium containing no antibody. Cultures were examined after a 96 h culture period. Viability was assessed by trypan blue exclusion and uptake of methyl-[<sup>3</sup>H]thymidine 5'-triphosphate ([<sup>3</sup>H]thymidine; Amersham International, Amersham, UK) was used to monitor the rate of cell division. Relative [<sup>3</sup>H]-thymidine uptake for  
30 each antibody dose was calculated by expressing the mean cpm incorporated (from triplicate wells) as a percentage of the average cpm incorporated in the wells containing no antibody.



*Determination of cell viability*

Cells dissociated by trypsin, were mixed with an equal volume of 0.1% w/v trypan blue in PBS and spread onto a haemocytometer. Cell viability was calculated as the percentage of cells excluding the dye.

5 *Determination of [<sup>3</sup>H] Thymidine uptake*

After 80 hours incubation, cells were then cultured for a further 16 hours with 0.5  $\mu$ Ci [<sup>3</sup>H] thymidine per well. After incubation the supernatant medium of adherent cells was removed and each well was washed twice with warm DMEM. Acid precipitable material was separated by  
10 addition of 250  $\mu$ l ice cold 5% w/v trichloroacetic acid (TCA, BDH Chemicals, Australia Pty Ltd. Kilsyth, Victoria, Australia) to each well (Plate, 1974, J. Exp. Med. 139 851-861). The precipitate was washed twice with TCA and solubilized in 0.3 ml 0.25 N NaOH; 250  $\mu$ l of this preparation was mixed with 2 ml scintillation cocktail (Emulsifier safe, Packard Instruments  
15 Co., Meriden, CT, USA) and cpm incorporated into acid precipitable material were determined for each well by  $\beta$ -counting.

*Immunocytochemistry*

Human T-cell leukaemia cells Molt 4 (ATCC CRL 1582) were maintained in log phase in RPMI + 10% FCS. Cells were washed three  
20 times in RPMI + FCS and incubated ( $10^6$  cells) with 10  $\mu$ g (in 0.1 ml) affinity purified anti-N peptide Ab, anti-I peptide Ab or control antibody (anti-ovalbumin Ab). Control tests contained  $10^6$  normal spleen cells. Bound antibody was detected by anti-rabbit biotinylated IgG, F(ab')<sub>2</sub> fragment (Amersham), followed by streptavidin-fluorescein according to the  
25 manufacturer's instructions. Binding was visualized by UV microscopy.

RESULTS

Tumour cell growth is perturbed by co-culture with anti-cpn10-derived peptides Abs.

Incubation of B16 melanoma and MCA-2 fibro sarcoma cells in  
30 increasing concentrations of anti-peptide Abs resulted in a significant decrease of cell division and increased cell death after 96 h incubation (FIGS. 9a, 9b; 10a, 10b). Incubation of cells in similar concentrations of control Ab had no

effect (FIGS. 9c, 10c).

Anti-I peptide antibody bound to cpn10 in the surface of Molt 4 cells. No binding was detected with anti-N peptide or anti-ovalbumin antibody on Molt 4 cells or with any of the abovementioned antibodies on normal spleen cells. This is the first visualization of extracellular cpn10 (FIG. 11).

#### CONCLUSION

The studies described here have established that anti-cpn10-derived peptides Abs inhibit the growth of tumour cells. The anti-proliferative effect of culturing B16 and MCA-2 cell lines in increasing doses of anti-peptides Abs is evidence that the growth of these cells is dependent upon continued presence of cpn10. These studies have established that the tumour cells require cpn10 for proliferation *in vitro*.

#### OTHER ASPECTS OF THE INVENTION

The abovementioned N-terminal fragment and internal fragment are regions of the molecule which are active in the rosette inhibition test and therefore function as active centres.

Pharmacological antagonists can be constructed, using conventional means, by modification of the structure of these active centres, so that binding to target sites, e.g. tumour cells, may occur without target activation. By interfering with the action of the whole cpn10 molecule on tumour cells, these antagonists will mimic the anti-proliferative effect described above for anti-cpn10 antibodies.

The invention also includes within its scope an assay for measuring anti-cpn10 antibody in a sample including the steps of:-

- (1) reacting substantially purified cpn10 with the sample; and
- (2) determination of the amount of anti-cpn10 antibody in the sample by determining the binding between the antibody and cpn10.

It will also be appreciated from the foregoing that data is available (e.g. Quinn *et al.*, 1992, Cancer Immunol. Immunother. 34 265-271) that anti-EPF antibodies are useful in suppression of tumour growth in a mouse model. Such data supports the assertion that anti-EPF antibodies will

suppress tumour growth *in vivo* or *in vitro*.

The dosages utilised in the administration of antagonists or antibodies are in the range of 1-1000 (more preferably 50-200)  $\mu\text{g/kg}$  of body weight for antagonists and 1-1000 (more preferably 50-200)  $\text{mg/kg}$  of body weight for antibodies. These dosages are based on a molecule which has the same molecular weight as cpn10 and dosages should be adjusted accordingly.

TABLE 1

Ag	Dose ( $\mu$ g/injection)
I-peptide	200
N-peptide	400
Ovalbumin	500

TABLE 2

		TITRE 1 log <sub>2</sub> reciprocal serum dilution		
Anti-serum	Test antigen	Pre Column (serum before column)	Bound (fractions)	Unbound (serum after column)
Anti-N ovalbumin	N-peptide	15	18	11
Anti-ovalbumin	I-peptide	13	19	10
Anti-ovalbumin	Ovalbumin	19	25	> 17

TABLE 3

Antibody (total dose 2 mg/ mouse)	No. of animals in group	Corpora lutea/ mouse (mean $\pm$ sem)	Embryo/ mouse (mean $\pm$ sem)	p*
Anti-N- peptide- ovalbumin	6	19.1 $\pm$ 1.2	10.6 $\pm$ 3.8	< 0.05
Anti-I- peptide- ovalbumin	6	20.8 $\pm$ 0.8	17.1 $\pm$ 1.1	< 0.02
Anti- ovalbumin	5	17.8 $\pm$ 1.0	16.8 $\pm$ 0.5	NS

**TABLE LEGENDS****TABLE 1**

Dose of antigen per injection administered to rabbits in the preparation of antibodies.

**TABLE 2**

Titre of affinity purified anti-cpn10 peptide antibodies and control anti-ovalbumin antibodies.

**TABLE 3**

Effect of passive immunization of confirmed-mated mice at days 1 and 2 *p.c.*, with antibodies to cpn10-derived peptides, on the number of implanted embryos and corpora lutea present at day 7 *p.c.*

\* (Heteroscedastic t-test).

## FIGURE LEGENDS

### **FIG. 1a**

Purification of EPF. Heat extracted human platelets (100 units) were fractionated on SP-Sephadex and Heparin Sepharose, then applied to a TSK-Phenyl 5PW column and eluted with a reverse salt gradient. Fractions were tested in the rosette inhibition test (based on EPF's capacity to augment the rosette inhibiting activity of an immunosuppressive antilymphocyte serum).

### **FIG. 1b**

Active fractions (n) from (a) were fractionated by RP-HPLC-1.

### **FIG. 1c**

Active fractions (n) from (b) were fractionated by RP-HPLC-2.

### **FIG. 1d**

Active fractions (n) from (c) were fractionated by RP-HPLC-3.

### **FIG. 1e**

Interaction of immobilised monoclonal anti-EPF antibody 5/341 with active fractions from (d) and equivalent fractions from human pregnancy serum, 6 d gestation (10 ml); human pregnancy urine, up to 1 month gestation (10 litre); medium conditioned by oestrous mouse ovaries (100) stimulated with prolactin and mouse embryo-conditioned medium (ovary CM); serum free medium conditioned by the bovine kidney cell line MDBK (MDBK-CM; ATCC CCL 22, 10 litre); rat serum obtained 24 h post-partial hepatectomy (post-pH, 10 ml); rat liver obtained 24 h post-pH (40 g); all fractionated as in (a) to (d). Anti-EPF bound and unbound fractions were tested in the rosette inhibition test, specificity was demonstrated by comparison with a parallel experiment using irrelevant antibody in which activity was not bound.

### **FIG. 2a**

Analysis of EPF purified from 300 units human platelets as in FIG. 1A. Determination of monomeric size. Iodinated EPF was fractionated by SDS-PAGE,<sup>29</sup> the gel sliced (2 mm wide slices) and the distribution of radioactivity and biological activity compared. (Inset) Direct Coomassie Blue staining of the same preparation.

### **FIG. 2b**

Ion-spray mass spectrum of EPF, displayed as multiply protonated molecular ions. (Inset) Computer reconstruction as molecular mass.

**FIG. 2c**

Amino acid sequence (single letter code) of peptides derived from human EPF, compared with rat cpn10 (underlined). EPF was digested with endoproteinase lys C and endoproteinase glu C, the resultant peptides separated by RP-HPLC and sequenced. The sequence of individual fragments is shown; all except 74-101 were derived from the lys digest.

**FIG. 3**

Interaction of EPF and cpn60 (*groEL*).

**FIG. 3a**

Peak fractions in the excluded volume of a TSK G3000SW gel permeation column, following application of a cpn60-EPF mixture +  $Mg^{2+}$ ATP, were analysed by SDS-PAGE (Schagger *et al.*, 1987) and stained with silver (Morrissey, 1981). Left lane, +ATP; right lane -ATP. (Cpn60 is a deca-tetramer, M, 840 000; column exclusion limit >300 000. Higher  $M_r$  bands on SDS gel are oligomeric forms of *groEL*).

**FIG. 3b**

Immobilised cpn60 was mixed with human pregnancy serum (6 d gestation) in the presence or absence of  $Mg^{2+}$ ATP. Unbound and bound fractions (the latter recovered from the gel by removal of ATP with EDTA) were then tested in the rosette inhibition test. Results are expressed as limiting dose, the highest dilution of sample giving a positive result in the rosette inhibition test.

**FIG. 4a, FIG. 4b, FIG. 4c**

Rabbit antibodies to cpn10-peptide/ovalbumin conjugates. Antibodies tested in an ELISA against immunising antigens.

**FIG. 5**

Anti-N-peptide, anti-EPF #816, anti-EPF #810 and control anti-ovalbumin antibodies (100 ng/ml) were tested in an ELISA against □ N-peptide (5  $\mu$ g/ml) and □ EPF/cpn10 (1  $\mu$ g/ml). Bound IgG was detected by the biotin-streptavidin system (Amersham) with o-phenylene diamine as substrate. Absorbance was read at 492 nm.



FIG. 6a

pRM1

FIG. 6b

pRM2

FIG. 6c

pRM3

FIG. 7

Preparation of antibodies to cpn10. Fusion protein (GST:rcpn10).

FIG. 8

Detection of anti-cpn10 antibodies in rabbit serum by ELISA. Serum harvested after the 4th booster dose of antigen.

FIG. 9

Relative [ $^3\text{H}$ ]thymidine uptake (—■—) and viability (--□--) of B16 melanoma cells after incubation for 96 h with anti-cpn10-derived peptide antibodies. Proliferation was assessed by uptake of [ $^3\text{H}$ ]thymidine into cells incubated with antibody, expressed as a percentage of [ $^3\text{H}$ ]thymidine incubated without antibody.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , (Student's  $t$  test)  $n = 3$ .

FIG. 10

Relative [ $^3\text{H}$ ]thymidine uptake (—■—) and viability (--□--) of MCA-2 fibrosarcoma cells after incubation for 96 h with anti-cpn10-derived peptide antibodies. Proliferation was assessed by uptake of [ $^3\text{H}$ ]thymidine into cells incubated with antibody, expressed as a percentage of [ $^3\text{H}$ ]thymidine incubated without antibody.

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , (Student's  $t$  test)  $n = 3$ .

FIG. 11

Anti-cpn10 I-peptide Abs detect cpn10 on the surface of human Molt 4 leukaemia cells.

CLAIMS

1. An antagonist to or antibody raised against cpn10 or a recombinant cpn10 which has the amino acid sequence GSAGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVEA VGSGSKGKGGEIQPVSVKEGDKVLLPEYGGTKVVLDDKDYFLFRDGDIL GKYVD.

2. An antagonist to or antibody raised against a peptide derived from cpn10.

3. An antagonist to or antibody raised against a peptide with an amino acid sequence Ac-AGQAFRKFLPLC, AGQAFRKFLPLC or EKSQGKVLQATC wherein said peptide may have a single amino acid deletion, addition or substitution.

4. An antagonist to or antibody raised against the following peptides:-

- (i) AGQAFRKFLPL;
- (ii) Ac-AGQAFRKFLPL;
- (iii) EKSQGKVLQAT
- (iv) A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub>;
- (v) AGQAFRKFLPLA<sub>2</sub>;
- (vi) A<sub>1</sub>AGQAFRKFLPL;
- (vii) Ac-A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub>;
- (viii) Ac-AGQAFRKFLPLA<sub>2</sub>;
- (ix) Ac-A<sub>1</sub>AGQAFRKFLPL;
- (x) A<sub>1</sub>EKSQGKVLQATA<sub>2</sub>;
- (xi) EKSQGKVLQATA<sub>2</sub>;
- (xii) A<sub>1</sub>EKSQGKVLQAT;

wherein A<sub>1</sub> and A<sub>2</sub> are amino acid sequences which may be added to one or each end of peptides (i) through (xii) and wherein said peptide(s) may have a single amino acid deletion, addition or substitution.

5. A method for suppressing cellular growth or enhancing immunological activity including the step of administration of a cpn10 antagonist or anti-cpn10 antibody to a subject.

6. A method as claimed in Claim 5 for terminating pregnancy including the step of administration of a cpn10 antagonist or an anti-cpn10 antibody to a subject.
7. A method as claimed in Claim 5 for suppressing cell growth including the step of administration of a cpn10 antagonist or anti-cpn10 antibody.
8. A method as claimed in Claim 7 wherein the cell is a tumour cell.
9. A method as claimed in Claim 8 wherein the cell is a leukaemia cell.
10. An assay for measuring anti-cpn10 antibody in a sample including the steps of:-
  - (1) reacting substantially purified cpn10 with the sample; and
  - (2) determination of the amount of anti-cpn10 antibody in the sample by determining the binding between the antibody and cpn10.

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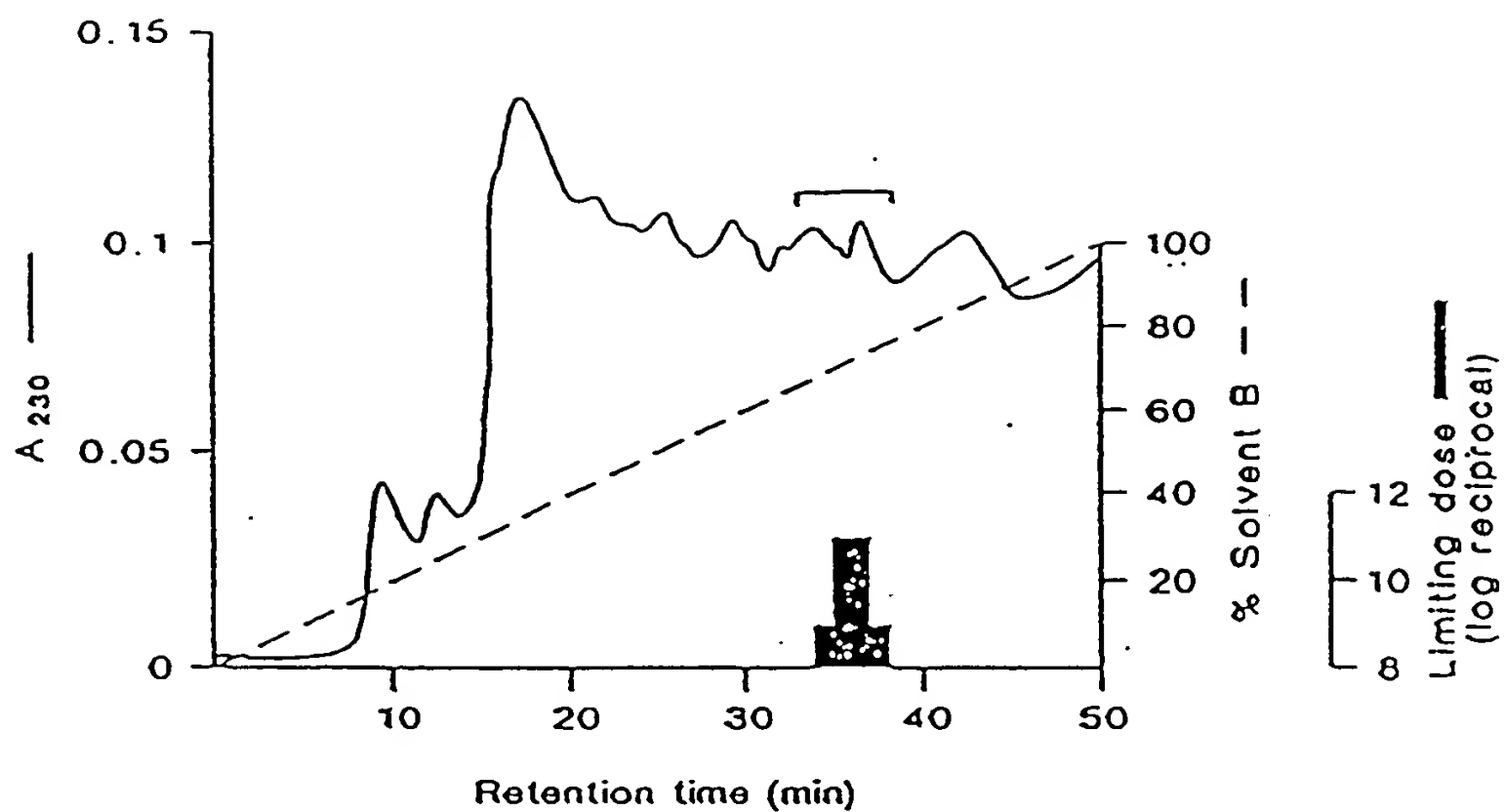


FIG 1a

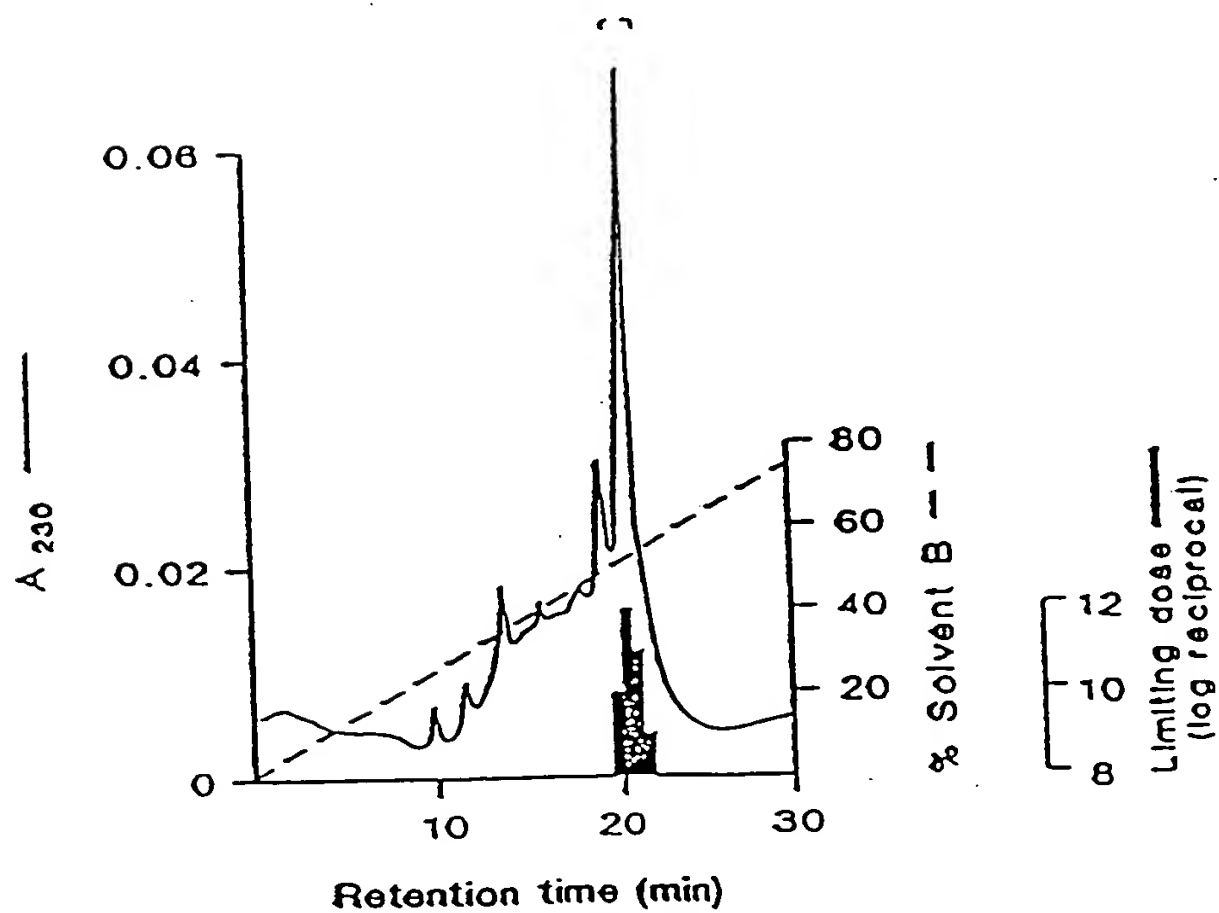


FIG 1b

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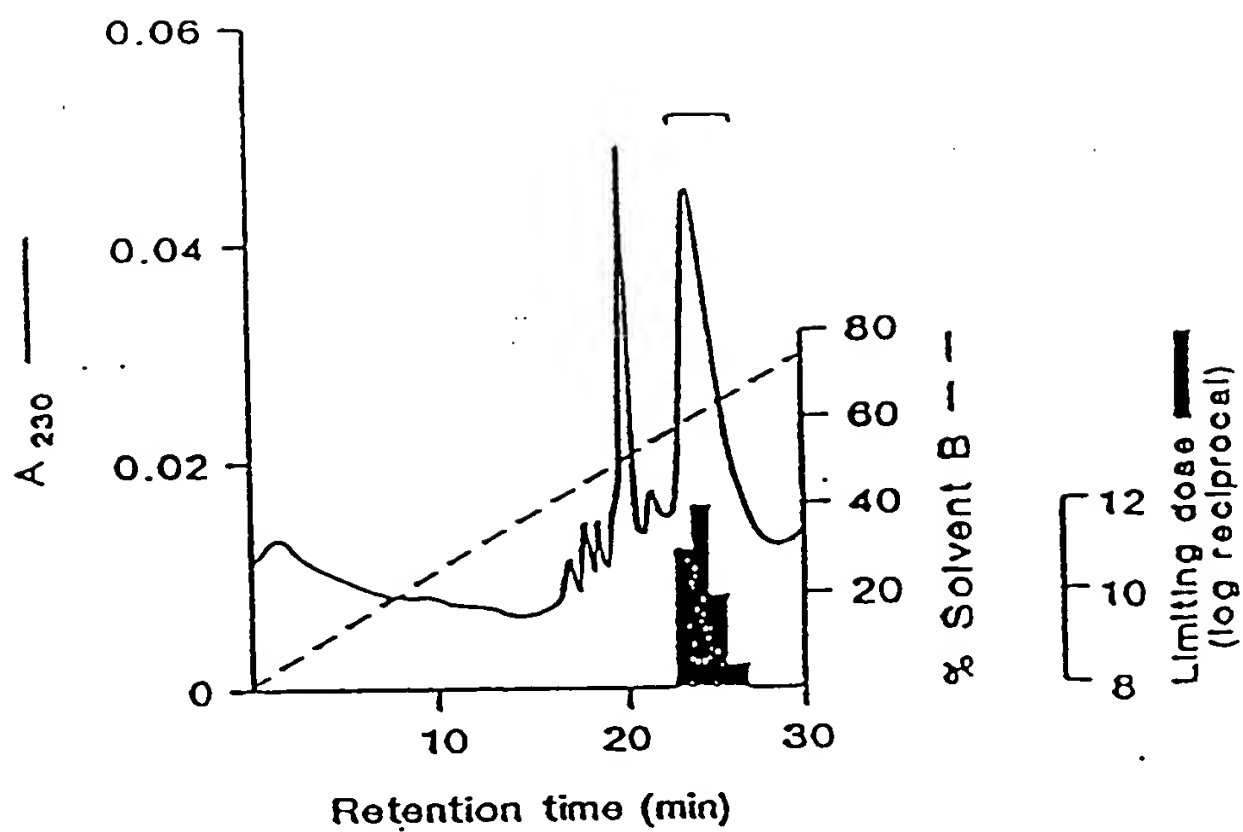


FIG 1c

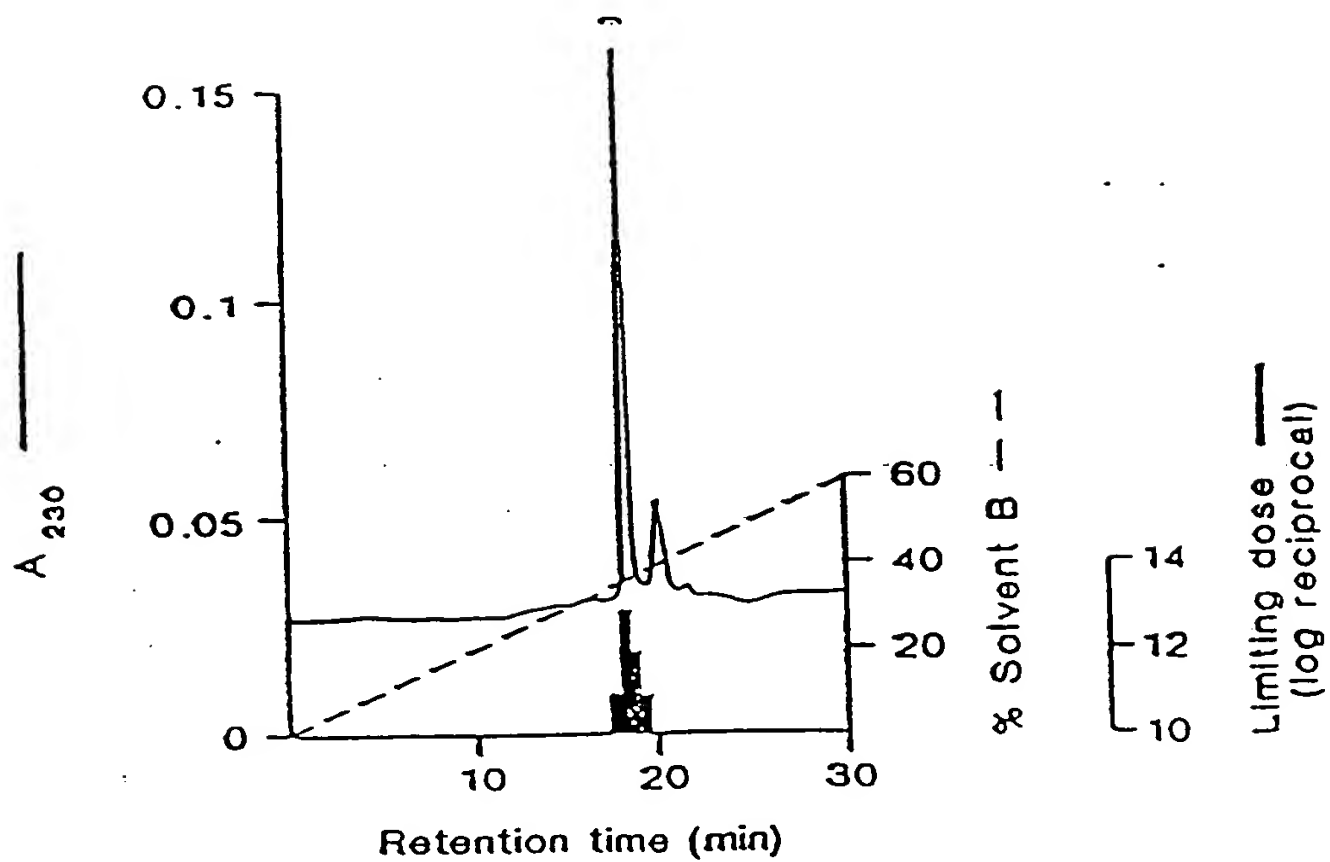


FIG 1d

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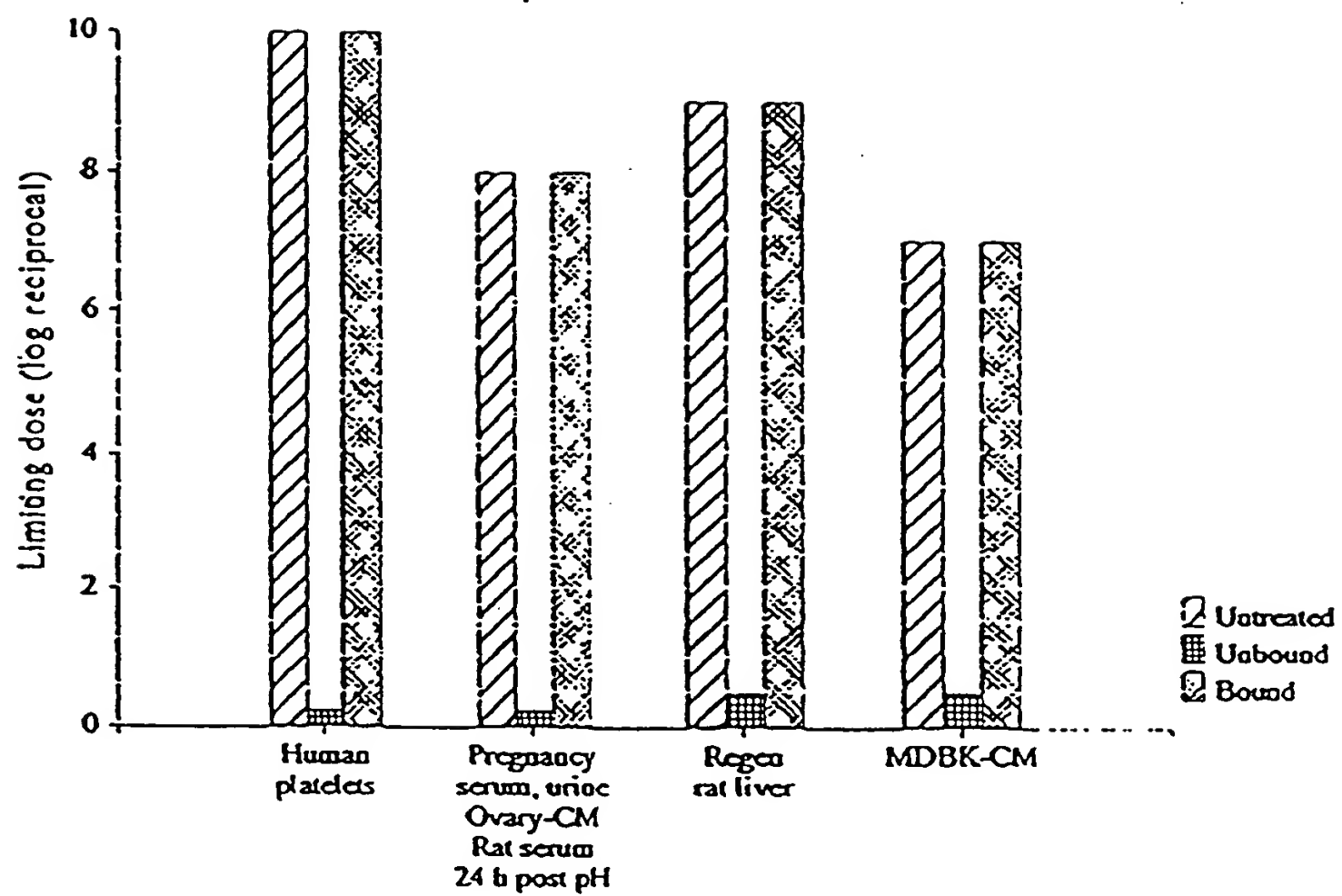


FIG 1e

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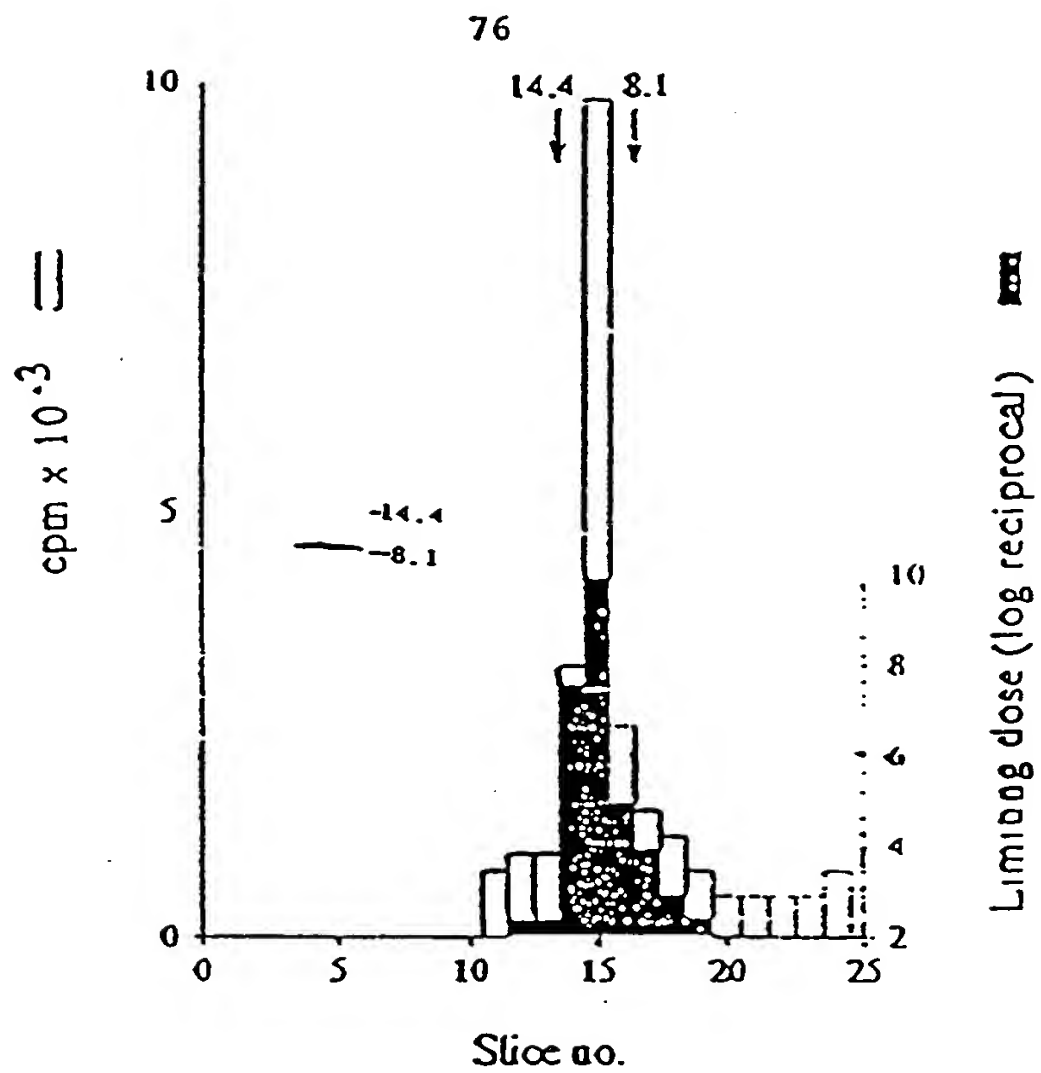


FIG 2a

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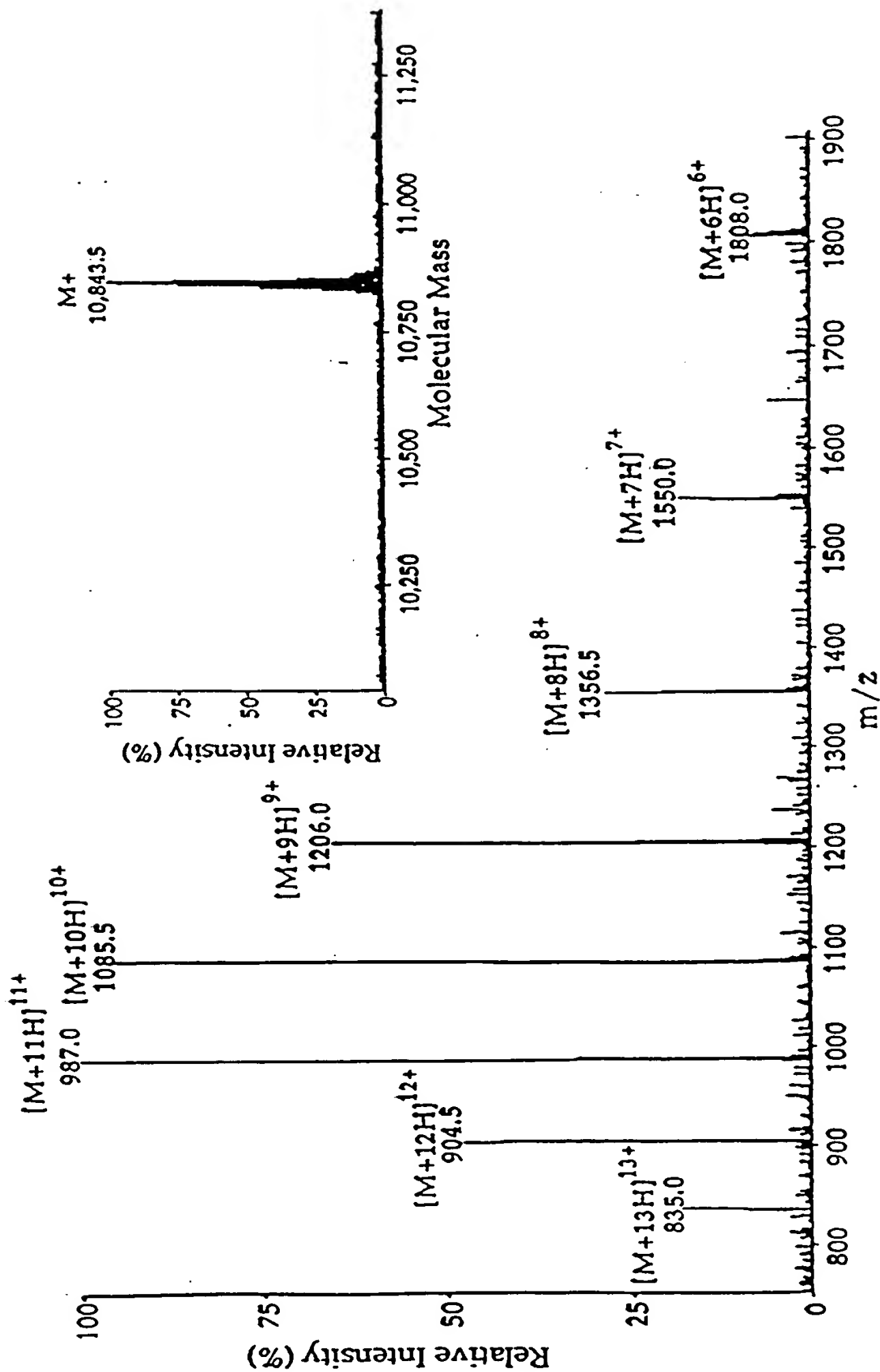


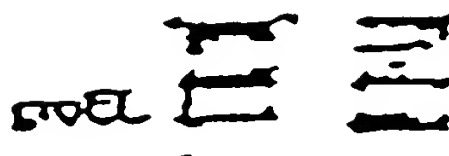
FIG 2b



Human EPF ) ) )	KFLP		LFDRVLVE		KQGI		KV		LXATVVAVOS	
	AC-(AQAQF)RXFLP		LFDRVLVERS		AAETVTKGGI		K3QGV		LQATVVAVGX	
	Rat cpn10		10		20		30		40	
Human EPF ) ) )	OSK		EYGGTKV		VXXXDXFLF		RQDILOKYV D			
	OX		LLPEYGGT		KV		VLDGDKDYFLF		RQDILOKYV D	
	Rat cpn10		60		70		80		90	
		GCKKGGGELQ		PXXXKXGXV		LLPEYGGTKV		VLDGDKDYFLF		RQDILOKYV D
										100

FIG 2c

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EPF —

WT WT

FIG 3a

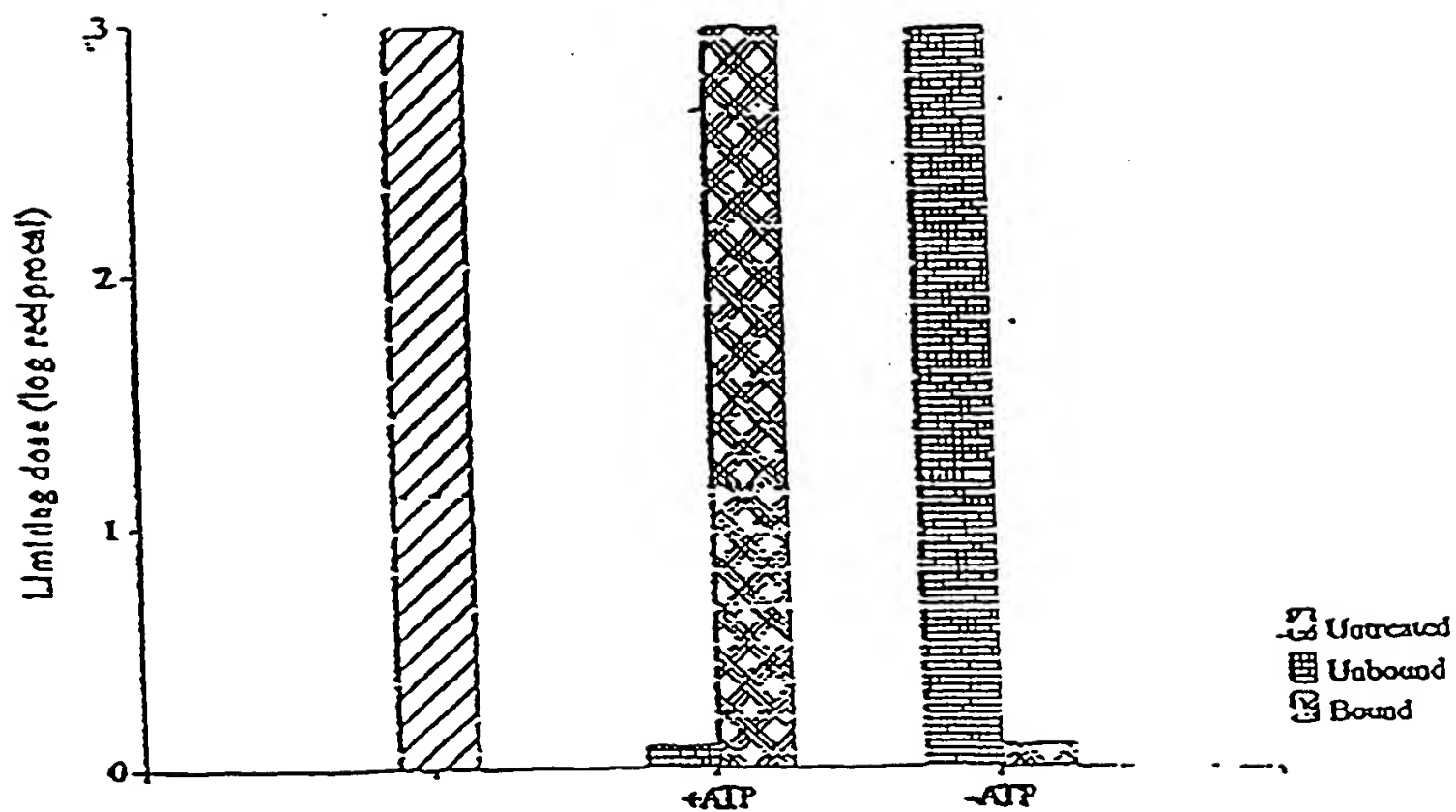


FIG 3b

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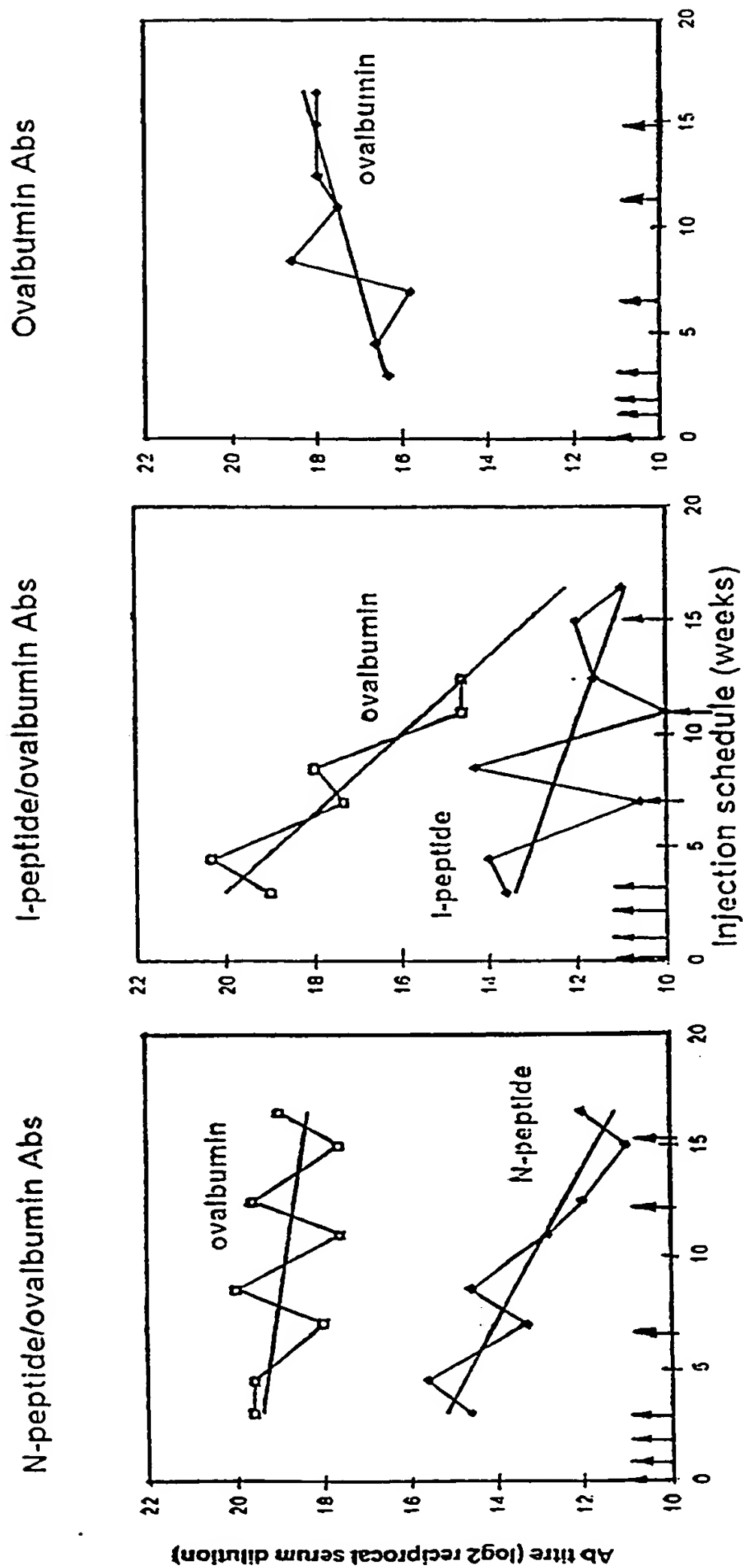


FIG 4c

FIG 4b

FIG 4a

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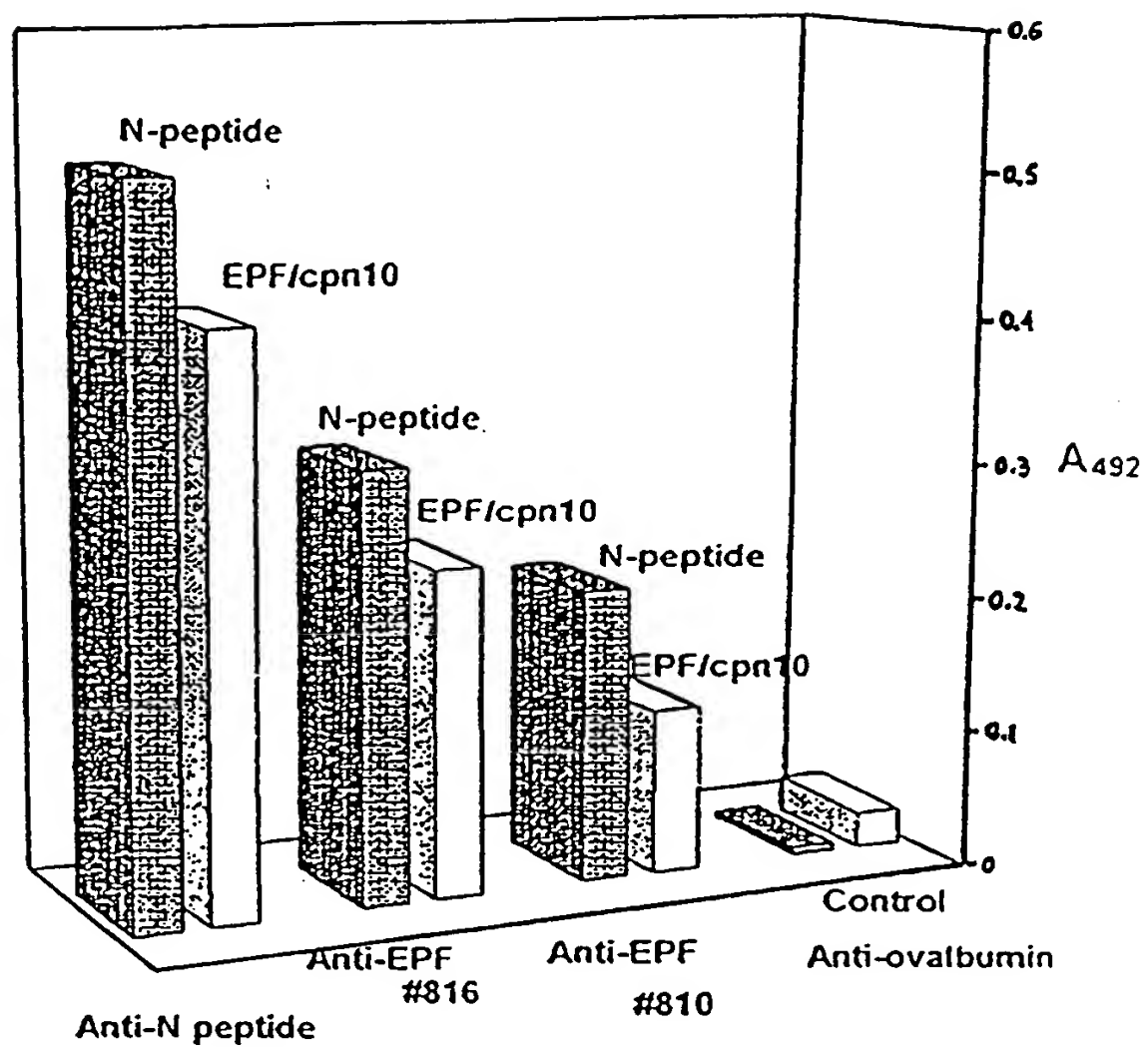


FIG 5

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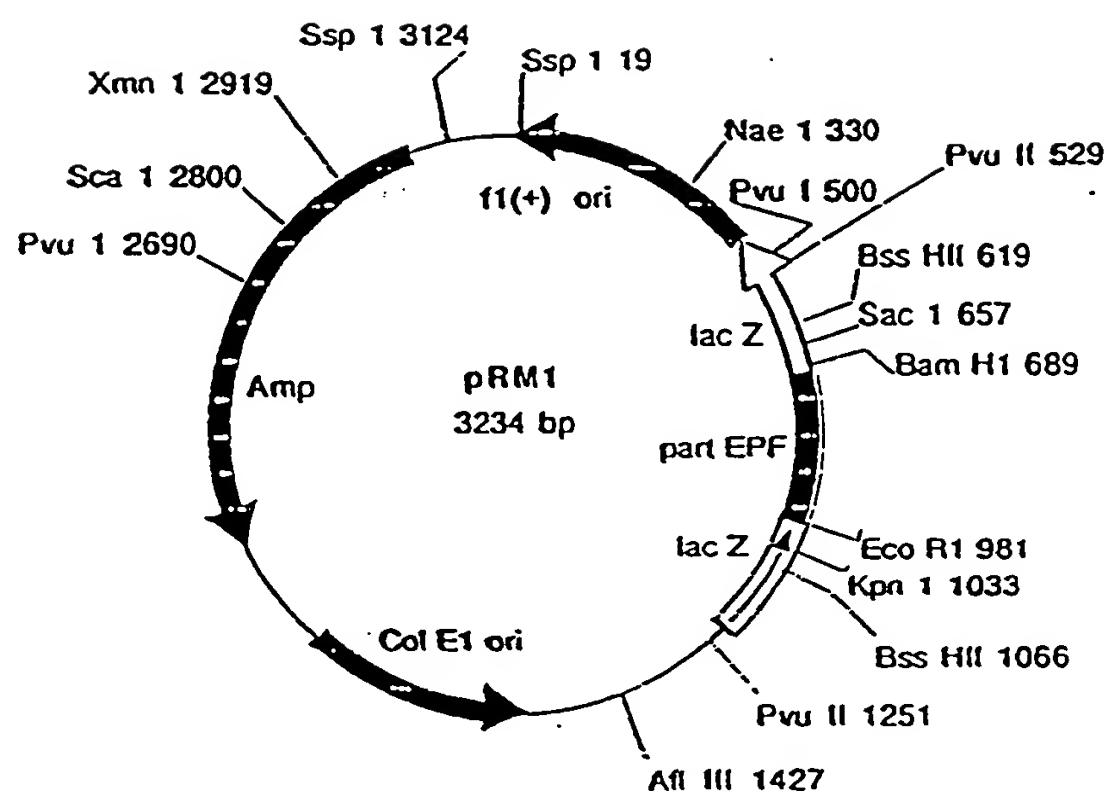


FIG 6a

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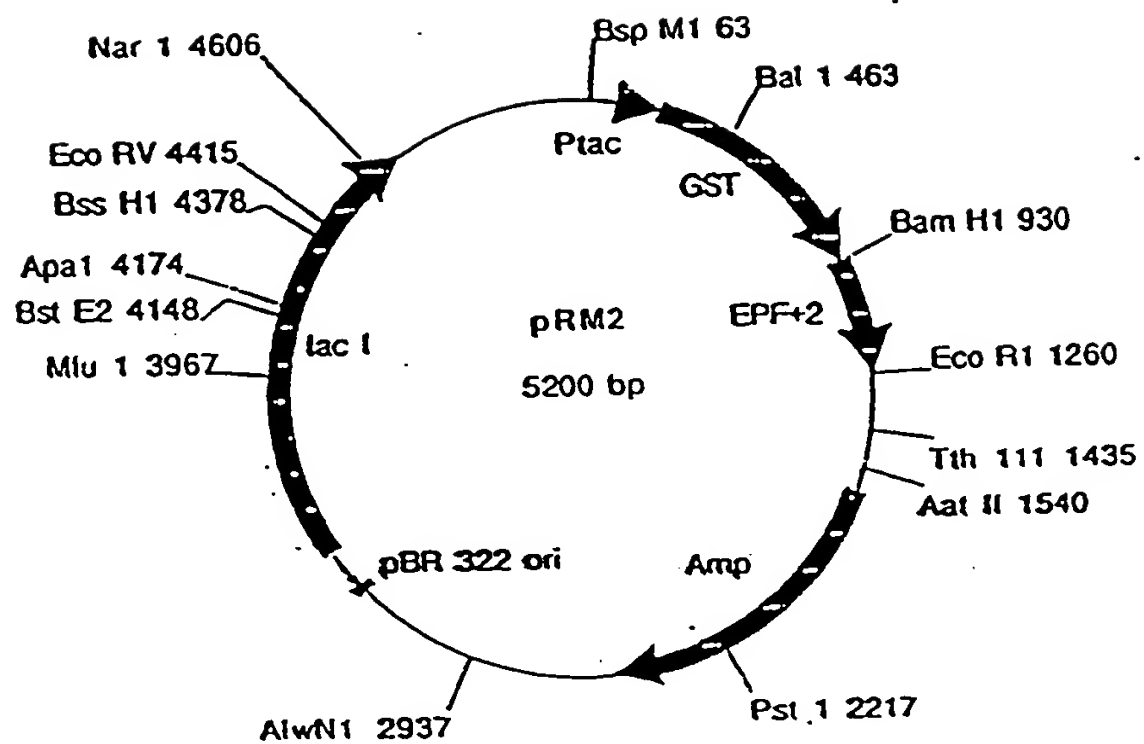


FIG 6b

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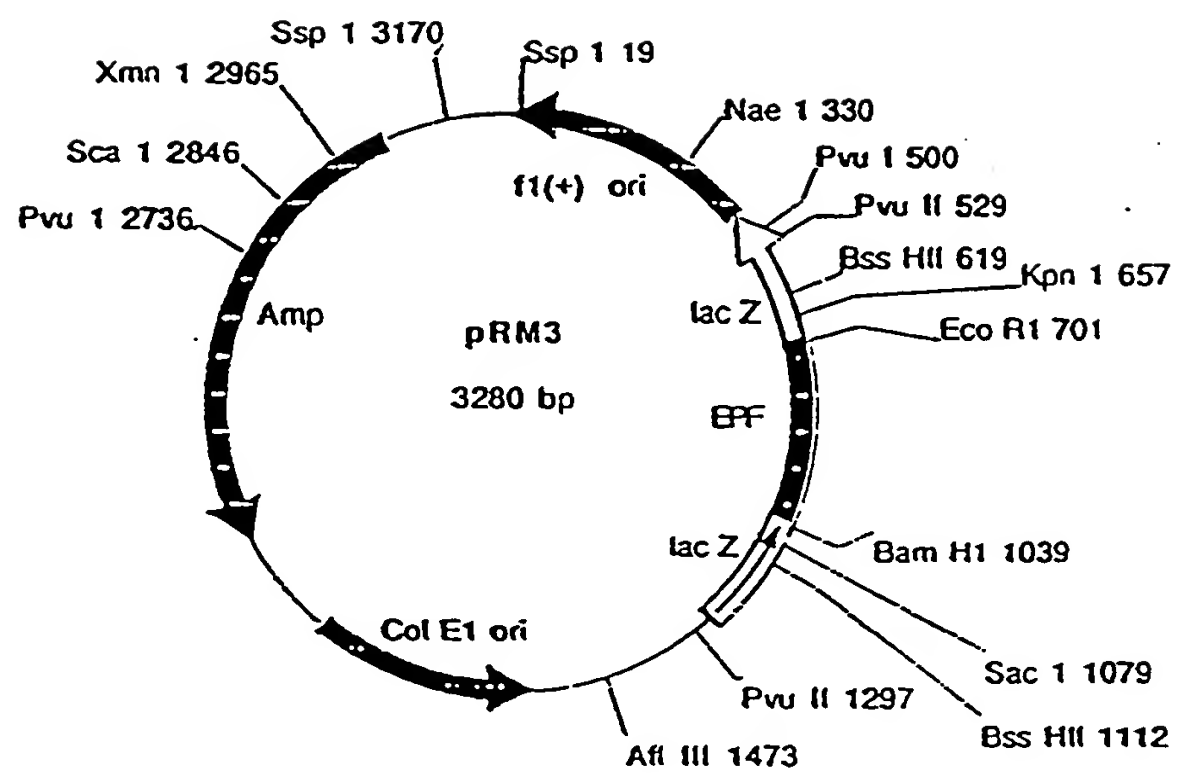


FIG 6c

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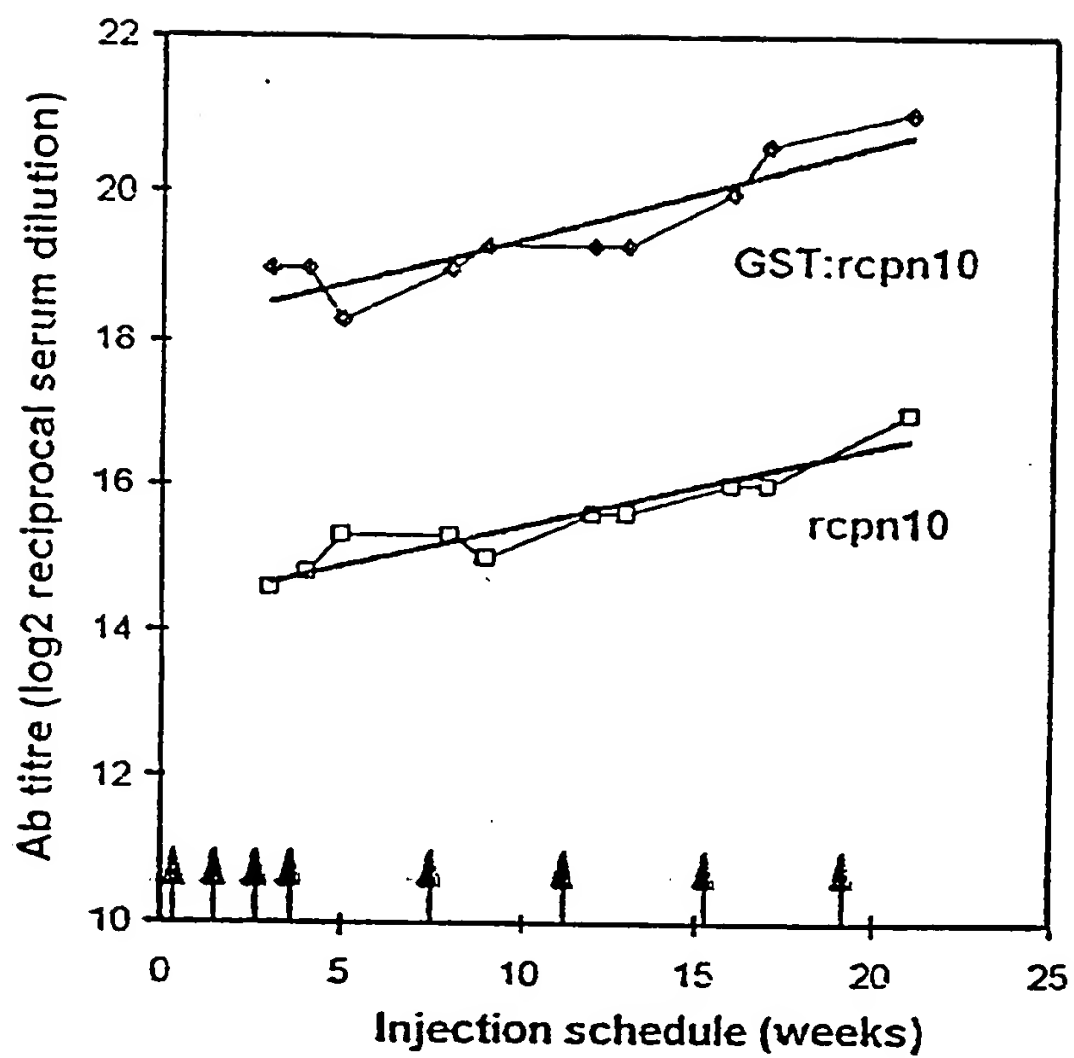


FIG 7



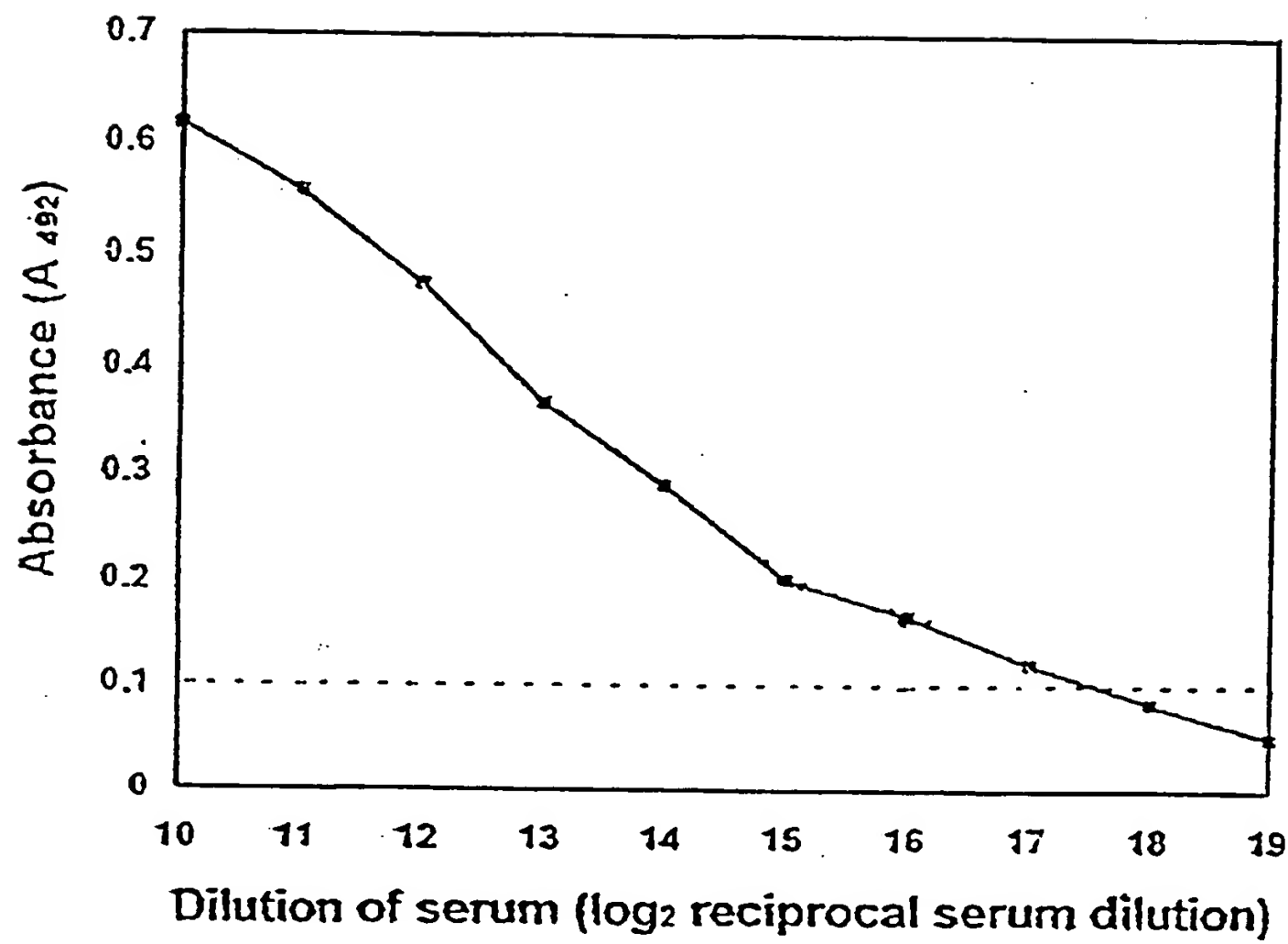


FIG 8

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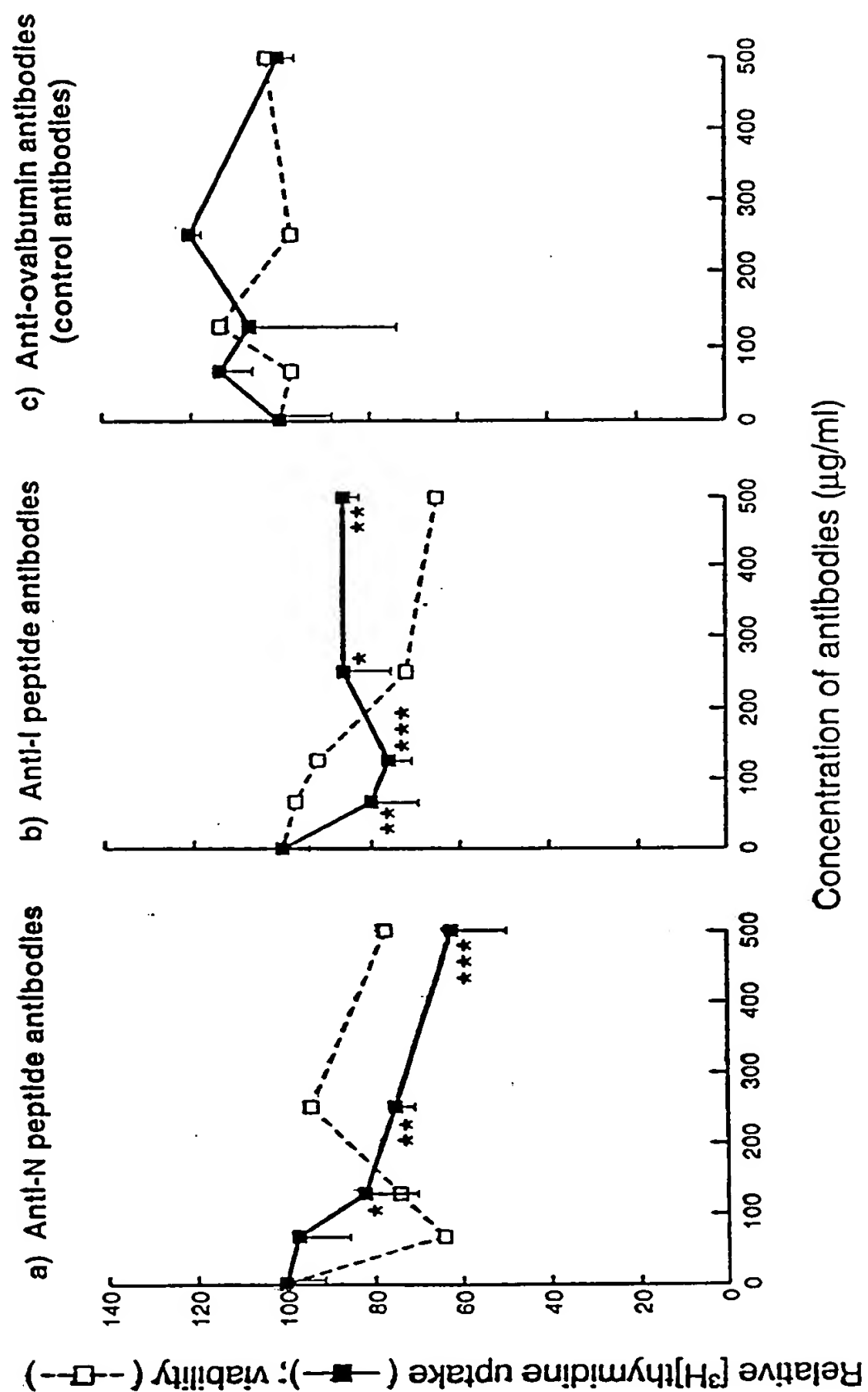


FIG 9

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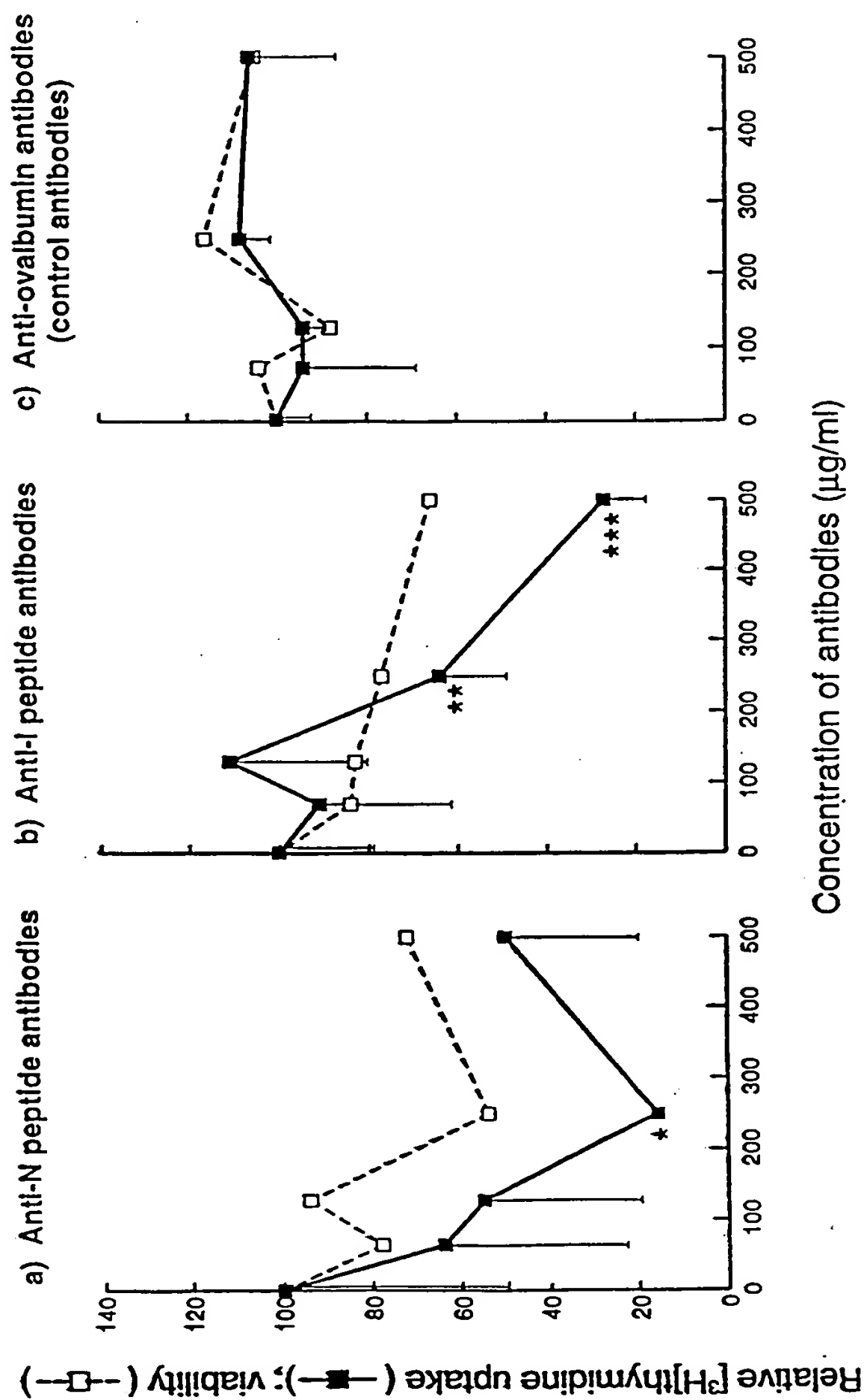


FIG 10

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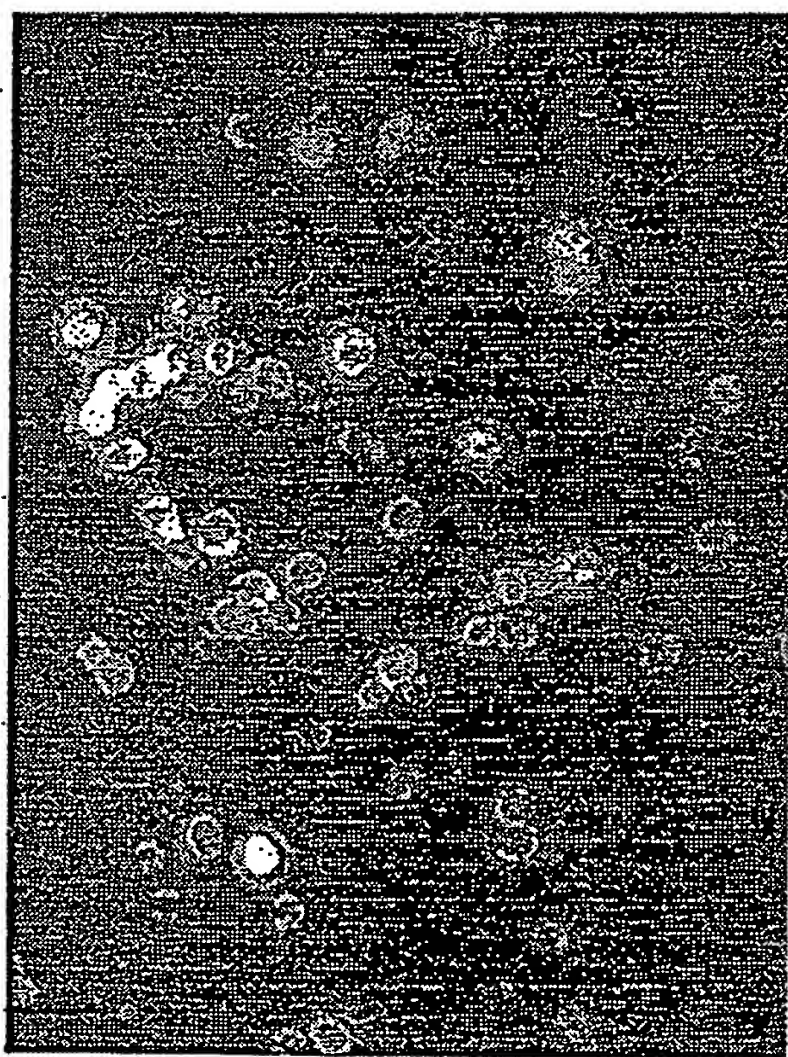



FIG 11

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU 94/00742

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>6</sup> C07K 16/18, G01N 33/68, A61K 38/08, 38/17  According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) See KEYWORDS used in electronic data base box below  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched BIOTECHNOLOGY (DERWENT DATABASE) MEDLINE KEYWORDS see electronic data base below  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT - WPAT, USPM, BIOT, CASM (CHEMICAL ABSTRACTS) )KEYWORDS "CHAPERONIN 10" )OR "EARLY PREGNANCY OR )PREGNANCY FACTOR"  STN INTERNATIONAL - The following protein subsequences: KFPLP OR EKSQG				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.		
P,X	European Journal of Biochemistry volume 222 pages 551-560 (1994) Cavanagh Alice C. and Morton Halle "The purification of early pregnancy factor to homogeneity from human platelets and identification as chaperonin 10". See specially column 2 of page 551	1		
X	Cancer Immunology and Immunotherapy (1992) volume 34 page 265-271 Quinn K.A. and Morton H. "Effect of monoclonal antibodies to early pregnancy factor (EPF) on the in vivo growth of transplantable murine tumors". See page 266-268	1-5, 7-9		
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;">           * Special categories of cited documents :            "A" document defining the general state of the art which is not considered to be of particular relevance            "E" earlier document but published on or after the international filing date            "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            "O" document referring to an oral disclosure, use, exhibition or other means            "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 50%; vertical-align: top;">           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention            "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone            "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art            "&amp;" document member of the same patent family         </td> </tr> </table>			* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search 13 March 1995 (13.03.95)	Date of mailing of the international search report 15 Mar 1995 (15.03.95)			
Name and mailing address of the ISA/AU  AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA  Facsimile No. 06 2853929	Authorized officer  J.H. CHAN  Telephone No. (06) 2832340			

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU 94/00742

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	Clinical experimental Immunology (1990) volume 80 pages 100-108 Quinn K.A. et al "Monoclonal antibodies to early pregnancy factor perturb tumour cell growth". See whole document	1, 5, 7-9
P,X	Hepatology volume 20 no. 5 (1994) pages 1294-1302 Quinn K.A. et al "Early Pregnancy Factor in Liver regeneration after partial hepatectomy in rats: Relationship with chaperonin 10" See entire document	1-4
X	Proceedings of National Academy of Science USA volume 89 pages 3394-3398 (1992) Hartman Dallas J et al "Identification of a mammalian 10K-Da heat shock protein, a mitochondrial chaperonin 10 homologue essential for assisted folding of trimeric ornithine transcarbamoylase in vitro".  This document (fig 5 especially) discloses cpn 10 homologue, which is inside the ambit of antagonist as defined in claim 1.	1
P,X	Biochemica et Biophysica Acta volume 1218, pages 478-480 (1994) Monzini Nicoletta et al "Identification and cloning of human chaperonin 10 homologue". See figure 3  Figure 3 of this document discloses cpn 10 homologues.	1
X	AU,B, 599021 (55897/86), University of Queensland 25 September 1986 (25.09.86)  This document discloses antibodies to early pregnancy factor.	1
X	AU,B, 10869/88 University of Queensland 30 June 1988 (30.06.88)  This document discloses antibodies to early pregnancy factor, and methods of suppressing tumor growth or terminating pregnancy by using these antibodies.	1
A	Semonirs in Reproductive Endocrinology volume 10 number 2 May 1992 Morton H et al "Early Pregnancy Factor".	

# INTERNATIONAL SEARCH REPORT

## Information on patent family memt

International application No.  
PCT/AU 94/00742

**This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.**

Patent Document Cited in Search Report			Patent Family Member				
AU	10869/88	EP	345260	WO	8804779		
AU	55897/86	EP	262119	GB	2192634	WO	8605498